Implication of β-endorphin released by activation of ascending pain pathways in attenuation of opioid reward in neuropathic pain states

Implication of β-endorphin released by activation of ascending pain pathways in attenuation of opioid reward in neuropathic pain states

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This dissertation is dedicated to my parents and my brother.

Table of Contents

Abbreviations

Chemical substances and drugs

CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2

DAMGO: [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*,-tetraacetic acid

FG: Fluoro-gold

GDP: Guanosine-5'-diphosphate

[^{35}S]GTPγS: Guanosine-5'-o-(3-[^{35}S]thio)triphosphate

PDBu: Phorbol 12,13-dibutyrate

PTX: Pertussis toxin

Buffers

PBS: Phosphate-buffered saline

Receptors

PAR: Protease-activated receptor

PDGFRα: PDGF α receptor

PDGFRβ: PDGF β receptor

Endogenous substances

BDNF: Brain-derived neurotrophic factor

PDGF: Platelet-derived growth factor

POMC: Proopiomelanocortin

Enzymes and intracellular messengers

ERK: Extracellular signal regulated kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

p-cPKC: Phosphorylated-conventional PKC

PTK: Protein tyrosine kinase

TH: Tyrosine hydroxylase

p-TH: Phosphorylated-TH

Brain region

ACC: Anterior cingluate cortex

CG: Cingulate cortex

N.Acc.: Nucleus accumbens

SI: Primary somatosensory area

SII: Secondary somatosensory area

VTA: Ventral tegmental area

Injection route

i.t.: Intrathecal

s.c.: Subcutaneous

Instrument

CPP: Conditioned place preference

ECD: Electrochemical detection

HPLC: High-performance liquid chromatography

fMRI: Functional magnetic resonance imaging

PET: Positron emission tomographic

RT-PCR: Reverse transcription-polymerase chain reaction

SPET: Singl photon emission tomographic

Others

ANOVA: Analysis of variance

BOLD: Blood oxygen level-dependent

rCBF: Regional cerebral blood flow

CNS: Central nervous system

CSF: Cerebro spinal fluid

DRG: Dorsal root ganglion

IR: Immunoreactivity

NIH: National Institute of Health

NeuN: Neuronal nuclei

ROIs: Regions of interest

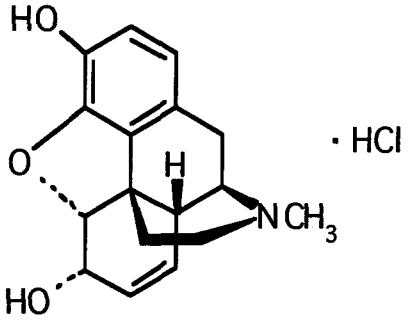
S.E.M.: Standard error mean

Ser: Serine

WHO: World Health Organization

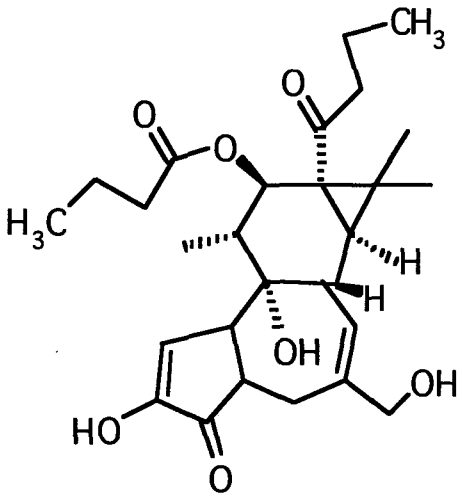
Structure of drugs used in the present study

Morphine hydrochloride

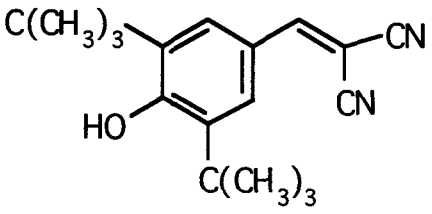


DAMGO: [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin

PDBu: Phorbol 12,13-dibutyrate



AG17: [(3,5-di-tert-butyl-4-hydroxybenzylidene)-malononitrile]



General Introduction

Neuropathic pain

Neuropathic pain, which is characterized by spontaneous burning pain, hyperalgesia (exaggerated pain in response to painful stimuli) and allodynia (pain caused by normally innocuous stimuli), is the most difficult pain to manage in the pain clinic. Since general analgesics such as non-steroidal anti-inflammatory drugs and opioids often fail to improve neuropathic pain, it is widely accepted that complicated mechanisms may underlie this pain syndrome. A growing body of evidence suggests that sensory nociceptive processing in the spinal dorsal horn appears to undergo significant plastic changes following peripheral nerve injury, leading to the development of neuropathic pain [1]. To date, although many studies have focused on the long-term changes in the functions of spinal cord dorsal horn neurons after nerve injury, the mechanisms of the development and maintenance of neuropathic pain are still not well understood.

Brain-derived neurotrophic factor/Platelet-derived growth factor

A considerable amount of evidence suuports the notion that neurotrophins may be involved in the modulation of synaptic plasticity. It is well known that immunostaining for another neurotrophin, brain-derived neurotrophic factor (BDNF), which is normally synthesized in small- to medium-diameter sensory dorsal root ganglia (DRG) neurons, and the level of BDNF mRNA are increased in ipsilateral DRGs

following sciatic nerve lesion [2,3]. In addition, the available evidence to date suggests that BDNF may be released from primary afferent neurons in an activity-dependent manner as an endogenous neurotransmitter/ neuromodulator [4-7].

Platelet-derived growth factor (PDGF), which is a neurotrophin, is a major mitogen for fibroblasts, smooth muscle cells and other cells [8,9]. It is a dimeric molecule that consists of disulfide-bonded, structurally similar A- and B-polypeptide chains, which combine to form homo- and heterodimers [10]. The PDGF isoforms exert their cellular effects by binding to and activating two structurally related protein thyrosin kinase receptors, denoted the α-receptor and the β-receptor [11-13]. The activation of PDGF receptors leads to the stimulation of cell growth, but also to changes in cell shape and motility: PDGF induces reorganization of the actin filament system and stimulates chemotaxis, i.e., a directed cell movement toward a gradient of PDGF [14-17].

Recent studies have demonstrated that PDGF A-chain mRNA is found in neurons in spinal cord and dorsal root ganglia of both embryoa and adult animals [18]. PDGF B-chain protein is also found in neurons in several central nervus system (CNS) regions in both the embryo and adult [19]. Considering these findings, I can speculate that PDGF within the spinal cord plays an important role in various critical physiological responses including pain perception. Thus, it is worthwhile to investigate the role of PDGF as well as BDNF in the modulation of synaptic plasticity linked to chronic pain.

Protein kinase C

Several protein kinases, such as protein kinase A (PKA) and protein kinase C (PKC), phosphorylate their target proteins, leading to transcriptional and/or posttranslational changes which in turn produce various critical physiological responses including pain perception [1,20]. In addition, several lines of evidence have demonstrated that activated protein kinases play a key role in the modulation of synaptic plasticity [21-23].

PKC, which is activated by 1,2-diacylglycerol in the presence of Ca^{2+} and phospholipids, acts as a key enzyme for signal transduction in various physiological processes [20,21,24]. The protein phosphorylation catalyzed by PKC may strongly modulate various processes, such as the release of neurotransmitters, cell proliferation and differentiation, and potentiation and desensitization of several kinds of receptor systems [20,21,24]. Recent studies have revealed a family of closely related proteins that can be subdivided on the basis of certain structural and biochemical similarities: Ca^{2+}-dependent or conventional isoforms (PKCα, βI, βII and γ; cPKCs), Ca^{2+}-independent or novel isoforms (PKCδ, ε, η and θ; nPKCs), and atypical isoforms (PKCλ and ζ; aPKCs) [22,23].

PKCα, βI, βII, γ, ε δ and ζ isoforms have been identified in the brain and spinal cord. These various isoforms appear to be differentially distributed in the mammalian central nervous system, and show cell type- and cellular component-specificity [25,26]. Interestingly, phorbol 12,13-dibutyrate (PDBu), a specific PKC activator, when given intrathecally (i.t.), produced spontaneous nociceptive pain-like behavior and long-lasting thermal hyperalgesia associated with an enhancement of neuronal activity in some brain regions related to pain perception [27,28]. Thus, the activation of protein

kinases may be responsible for the production of central sensitization, the spread of pain sensitivity to areas outside of tissue injury and the development of pain in response to low-intensity stimuli [20,25,26].

Among these PKC isoforms, it has been reported that PKCγ-like immunoreactivity was clearly increased in the dorsal horn of the rat spinal cord following peripheral nerve injury [29]. Furthermore, a neuropathic pain-like state induced by nerve ligation was not caused in mice that lacked the PKCγ gene [30,31]. These findings strongly suggest that activated PKC in the spinal cord may result in central sensitization to nociceptive transmission, which leads to the development of neuropathic pain.

Pain ascending pathway

The primary afferent nociceptor is generally the initial structure involved in nociceptive processes. The primary afferent fibers associated with the transmission of nociceptive signals to the substantia gelatinosa in the dorsal horn can be grouped into two main categories: small myelinated (A-δ) and unmyelinated (C) sensory afferent fibers [32,33]. The neuropathic pain following nerve injury or tissue inflammation depends on both an increase in the sensitivity of these first synapses at the site of injury and an increase in the excitability of neurons in the CNS [25,34-36].

Second-order neurons ascend the spinal cord to terminate in many supraspinal structures throughout the brain stem, thalamus and cortex. In the thalamus, these systems are divided into two main groups such as the ventrobasal complex and intralaminar nuclei [36]. The former, which includes the ventral posterolateral nuclei

and ventral posteromedial nuclei, is involved in the sensory discriminative component of pain and further projects to the somatosensory cortex [36-38]. The latter, which includes the central medial nuclei and parafascicular nuclei, is associated with the affective motivational aspects of pain and projects to the cingulate cortex (CG) [36,38]. The manifestation of pain can be explained on the basis of neural substrates mediating sensory, affective, and nociceptive functions, as well as neurovegetative responses. While the sensory, discriminative–perceptive component permits spatial and temporal localization, physical qualification and intensity quantification of the noxious stimulus, the cognitive–affective component attributes emotional coloring to the experience, and is responsible for the behavioral response to pain [39]. Therefore, I believed it would be worthwhile to investigate the ascending nociceptive transmission from the dorsal horn to brain areas involved in the processing of noxious stimuli.

Use of opioids to treat chronic pain

The μ-opioid receptor serves as the principle physiological target for most clinically important opioid analgesics. It has been shown that μ-opioid receptor transduces signals through pertussis toxin (PTX)-sensitive Gi/Go proteins to inhibit adenylate cyclase, increase membrane K^+ conductance, reduce Ca^{2+} current [40] and activate a phospholipase C (PLC)-IP_3 pathway depending on the stimulation of βγ subunits [41-43].

According to the World Health Organization (WHO) guidelines for patients with moderate or severe pain, the typical μ-opioid receptor agonist morphine has been considered a "gold standard" for the treatment of moderate to severe cancer pain. In

1965, fentanyl, an anilidopiperidine-class μ-opioid agonist, was reported as a potent synthetic analgesic. Fentanyl has a high affinity for μ-opioid receptors and its analgesic activity is 50-100 times more potent than that of morphine. The low molecular weight and lipid-solubility of fentanyl make it suitable for delivery via a transdermal therapeutic system. On the other hand, oxycodone, which has been in clinical use for many years, is a semi-synthetic opioid analgesic derived from the naturally occurring alkaloid thebaine. It has been demonstrated that oxycodone has a structure and lipid-solubility comparable to those of morphine, and is comparable or only slightly inferior to morphine with regard to its analgesic potency [44]. In the clinic, it has been reported that the peripheral administration of oxycodone is effective for relieving some symptoms of pain in patients with neuropathtic pain. Thus, opioids, such as morphine, fentanyl and oxycodone, have been recommended as the drug of choice for the management of patients with chronic pain.

Opioid reward and mesolimbic dopamine system

μ-Opioid receptor agonists such as morphine and heroin have marked effects on mood and motivation. They produce euphoria in humans and function as positive reinforcers in various species, i.e., they maintain drug-seeking behaviors, which lead to their administration. The reinforcing effects of these drugs may become so marked that they become the primary stimuli that motivate behavior, with subsequent of compulsive drug-seeking or addiction [45-47].

Many studies have pointed to the mesolimbic dopaminergic system as the origin of

this effect. The mesolimbic dopaminergic system originates in the ventral tegmental area (VTA) of the midbrain and projects to nucleus accumbens (N.Acc.) that structures closely associated with the limbic system. It has been implicated in the rewarding effects of intracrainial self-stimulation, in natural rewards such as food and water intake, and in the action of drugs of abuse, including opioids [48,49]. It is well documented that μ-opioid receptors located in the VTA are critical for opioid reward. An electrophysiological study demonstrated that systemic administration of morphine elicits an increase in the firing rate of dopaminergic neurons in the VTA [50]. Furthermore, opioids have been shown to increase dopamine release and dopamine metabolites in the mesolimbic dopamine terminal fields [51-55]. Using the CPP paradigm, intra-VTA administration of morphine produces a rewarding effect [56], and this effect is blocked by either systemic naloxone [56] or intra-VTA injections of naloxone methiodide [57]. The morphine- or DAMGO-induced place preference is blocked by either dopamine antagonists or neurochemical destruction of the N.Acc. [58,59]. Additionally, intra-VTA injections of the μ-opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) or naloxone induce place aversion, and these effects are inhibited by 6-hydroxydopamine-induced lesions of the N.Acc. [45]. These findings indicate that dopamine-containing neurons of the midbrain VTA, an area that possesses high densities of μ-opioid receptors, play a critical role in the rewarding and aversive effects of opioids.

Morphine dependence under a chronic pain

Since relief from cancer pain was reported by the WHO to be achievable for most people with cancer, morphine has been used mostly for severe pain. In Japan, the annual consumption of morphine for medical purposes has increased. However, the present amount of morphine consumed per capita in Japan is still less than one-sixth of that in the United States [60]. This means that many patients still continue to suffer ongoing pain during the course of their illness. One major reason is that undue anxiety about psychological dependence on morphine in cancer patients has caused physicians and patients to use inadequate doses of opioids.

It has often been proposed that opioid addiction does not arise when opioids are used to treat pain. Some patients rarely show withdrawal signs when the pain is relieved and the opioid drug is gradually withdrawn. In fact, this argument has been used to support the safe use of opioids for the treatment of severe acute, cancer and chronic pain [61,62]. Indeed, animal and human studies support the contention that opioid euphoria and reward are attenuated by pain [63-68]. Interestingly, inflammatory nociception leads to the activation of endogenous κ-opioidergic systems in the brain [63,67,69]. On the other hand, neuropathic pain due to sciatic nerve ligation causes the dysfunction of μ-opioid receptor in the VTA [65,66]. These phenomena could be responsible for the suppression of the morphine-induced rewarding effect associated with the decrease in the release of dopamine in the brain of rodents. However, the mechanism of the suppression of opioid reward under chronic pain remains unclear.

Anatomy and imaging

Anatomical pathologists diagnose disease and gain other clinically significant information through the examination of tissues and cells. This generally involves gross and microscopic visual examination of tissues, with special stains and immunohistochemistry to visualize specific proteins and other substances in and around cells. More recently, anatomical pathologists have begun to use molecular biology techniques to gain additional clinical information from these same specimens.

Neuroimaging includes the use of various techniques to either directly or indirectly image the structure and function/pharmacology of the brain. Recent advances in functional neuroimaging techniques promise to promote our understanding of the dynamics of the human brain in the perception of pain, i.e., central processing involving sensation, affection, association and cognition. A series of positron emission tomographic (PET), single photon emission tomographic (SPET), and functional magnetic resonance imaging (fMRI) studies on experimentally elicited phasic thermal and chemical pain, using regional cerebral blood flow (rCBF) as an index of neuronal activity, have consistently demonstrated the activation of the primary and secondary somatosensory area (SI, SII), anterior cingulate cortex (ACC), thalamus, insula, prefrontal cortex and cerebellum [70-73]. These regions have thus been suggested to participate in the central processing of nociception. Therefore, it is considered that imaging techniques, such as immunohistochemistry and fMRI, may be used to reveal a relatively new discipline within medicine and neuroscience/psychology.

Aim and Scope

The aim of the present study was to investigate the mechanisms that underlie the suppression of opioid reward under a neuropathic pain-like state in rodents. Behavioral, neurochemical and biochemical experiments were conducted.

The specific aims of the proposed research are as follows:

In Chapter 1:

To clarify the role of a spinal PKC/thrombin/protease-activated receptor (PAR)/ /PDGF-mediated signaling pathway in the development of the neuropathic pain-like state induced by partial sciatic nerve ligation, I performed *in vivo* experiments using hirudin, which is a specific and potent thrombin inhibitor, PDGF α receptor (PDGFRα)/Fc chimera protein and the PDGFR-dependent PTK inhibitor. Furthermore, *in vitro* studies were performed to investigate whether the immunoreactivity to PAR-1 and binding of guanosine-5'-o-(3-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) to membranes of the spinal cord induced by thrombin could be affected by sciatic nerve ligation.

In Chapter 2:

To ascertain whether the direct activation of PKC in the spinal cord could change brain activation, I investigated whether intrathecal injection of the PKC activator

phorbol 12,13-dibutyrate (PDBu) could change the brain activation using the fMRI method in mice with a deletion of the gene that encodes the neuron-specific PKCγ isoform.

In Chapter 3:

To further examine whether activation of PKC in the spinal cord could lead to changes in the brain endogenous opioidergic system in the brain, I investigated whether a gene deletion for an endogenous μ-opioid peptide β-endorphin could affect pain-like behavior and the suppression of the morphine-induced rewarding effect by the direct activation of PKC in the spinal cord.

In Chapter 4:

To clarify the mechanism of the suppression of opioid reward under a chronic pain, I investigated whether released β-endorphin could affect the suppression of the opioid-induced rewarding effect, reduce μ-opioid receptor function and decrease the level of increased dopamine release in the nucleus accumbens under a neuropathic pain-like state using β-endorphin knockout mice.

Ethics

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study.

Chapter 1

Protease-activated receptor-1 and platelet-derived growth factor in spinal cord neurons are implicated in neuropathic pain after nerve injury

Introduction

Recently, our understanding of the role of serine proteases such as thrombin, a key enzyme of the coagulation system, has been expanded to include actions in the nervous system [74]. Thrombin affects protease-activated receptors (PARs), which are a family of G protein-coupled receptors [75]. Molecular cloning has identified four PARs. Thrombin activates PAR-1, PAR-3 and PAR-4. Thrombin cleaves PARs, unmasking the "tethered ligand", an extracellular N-terminal domain that subsequently binds and activates the receptor [76]. It is considered that PARs are implicated in responses to injury, notably in inflammation and repair [77]. In particular, PAR-1, which mediates most of the known proinflammatory actions of thrombin, is expressed by platelets, endothelial cells, fibroblasts, smooth muscle cells, mast cells, neurons and astrocytes [78]. Furthermore, PAR-1 is present in the developing and mature central nervous system [79]. Zhu et al. (2005) [80] reported that PAR-1 is expressed not only in a subset of large diameter primary sensory neurons, but also in a subset of small and medium diameter primary sensory neurons of the spinal cord which play an important role in the transmission of pain. The thrombin inhibitor hirudin, which is isolated from leech (*Hirudo medicinalis*), was characterized as a specific thrombin inhibitor in the late 1950s [81]. In addition, the high affinity of hirudin for thrombin and the specificity of the tight and essentially irreversible binding of hirudin to thrombin has been recently confirmed [82]. Therefore, hirudin is a useful tool for the elucidation of mechanisms of thrombin-induced physiological responses.

Recently, it has been reported that thrombin induces the activation of platelets and promotes the expression or the release of PDGF from α-granule of platelets [83]. PDGF was identified 20 years ago as a growth-promoting activity in human platelets for fibroblasts, smooth muscle cells, and glial cells. There are three PDGF isoforms (PDGF-AA, PDGF-AB, and PDGF-BB), which are homo- or heterodimers of related A and B polypeptide chains. Two receptors for PDGF (PDGF α receptor (PDGFRα) and PDGFRβ) bind the PDGF isoforms with different affinities. The PDGFRα binds all three PDGF isoforms with similar affinities. The binding of PDGF to PDGFR has been shown to induce homodimerization of the receptor, which facilitates the intrinsic protein tyrosine kinase (PTK) activity of PDGFR [84]. The activation of PTK autophosphorylates several tyrosine residues found within the cytoplasmic domain of PDGFR and provides a recognition site for intracellular signal molecules. Furthermore, it has been reported that PDGF and PDGFR could be located in myelinated and unmyelinated primary sensory neurons [85] and in the spinal cord [86]. Thus, these findings support the idea that PDGF, which is mostly present in the blood vessels, may play a more important role in the physiological responses including pain perception than has been thought previously. In addition, as described above, thrombin could release PDGF through PAR in platelets. Taken together, these findings raise the possibility that PDGF associated with PAR may be implicated in pain perception. However, the contribution of a thrombin/PAR/PDGF pathway to the neuropathic pain-like state is unknown.

In the present study, therefore, I investigated the role of a spinal

thrombin/PAR/PDGF-mediated signaling pathway in the development of the neuropathic pain-like state induced by partial sciatic nerve ligation in mice.

Materials and Methods

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd., Tokyo Japan) weighing about 25 g were housed at a room temperature of 23±1℃ with a 12 hr light/dark cycle (light on 8:00 am to 8:00 pm). Food and water available ad libitum during the experimental period.

Neuropathic pain model

The mice were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). A partial sciatic nerve injury by tying a tight ligature with 7-0 or 8-0 silk suture around approximately 1/3 to 1/2 the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30, Olympus, Tokyo, Japan) as described previously [30]. In sham-operated mice, the nerve was exposed without ligation.

Measurement of the latency of paw withdrawal in response to a thermal stimulus

To assess the sensitivity to thermal stimulation, each of the hind paws of mice was tested individually using a thermal stimulus apparatus (model 33 Analgesia Meter; IITC Inc./ Life Science Instruments, Woodland Hills, CA, USA). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 8 to 10 sec in naive mice. Only quick hind paw movements (with or without licking of hind paws) away from the stimulus were considered to be a

withdrawal response. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between the left and right with an interval of more than 3 min between measurements. The latency of paw withdrawal after the thermal stimulus was determined as the average of three measurements per paw. Before the behavioral responses to the thermal stimulus were tested, mice were habituated for at least 1 hr in an acrylic cylinder (15 cm high and 8 cm in diameter). Under these conditions, the latency of paw withdrawal in response to the thermal stimulus was tested. The latency of paw withdrawal in response to a thermal stimulus was measured before surgery and 1, 3, 5, 7 and 14 days after surgery.

To investigate the effect of a single i.t. treatment with thrombin or PDGF in normal mice, the latency of paw withdrawal was measured before and after injection until the latency returned to the baseline. The latency of paw withdrawal in response to a thermal stimulus was determined as the average of both paws.

Measurement of paw withdrawal in response to a tactile stimulus

To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA, USA) with two different bending forces (0.02 g and 0.16 g). Each von Frey filament was applied to the plantar surface of the hind paws for 3 sec, and this was repeated 3 times. Each of the hind paws of mice was tested individually. Paw withdrawal in response to a tactile stimulus was evaluated by scoring as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal

response away from the stimulus without flinching or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Paw withdrawal in response to each filament was determined as the average of two scores per paw. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between left and right with an interval of more than 3 min between the measurements. Before the behavioral responses to a tactile stimulus were tested, mice were habituated for at least 1 hr on an elevated nylon mesh floor. Under these conditions, paw withdrawal in response to a tactile stimulus was tested. The paw withdrawal threshold to the tactile stimulus was measured before surgery and the day after measurement of the thermal threshold (Day 2, 4, 6, 8 and 15). To investigate the effect of a single i.t. treatment with thrombin or PDGF in normal mice, the measurement of paw withdrawal responses to the tactile stimulus was performed before and after the injection until the response returned to the baseline. The paw withdrawal in response to the tactile stimulus was determined as the average of both paws.

Intrathecal injection

Intrathecal (i.t.) injection was performed as described by Hylden and Wilcox (1980) [87] using a 25-μL Hamilton syringe with a 30 1/2-gauge needle. The needle was inserted into the intervertebral space between L5 and L6 of the spinal cord. A reflexive flick of the tail was considered to be a sign of the accuracy of each injection. The injection volume was 4 μL for i.t. injection.

Groups of mice were repeatedly treated i.t. with the thrombin inhibitor hirudin (30 pmol/mouse), a recombinant mouse PDGFRα/Fc chimera protein (10 ng/mouse; PDGFRα/Fc) or the PDGF receptor-dependent tyrosine kinase inhibitor AG17 (30 nmol/mouse) 30 min, 1 hr or 30 min, respectively, before surgery and once a day for 8 consecutive days after surgery. A single i.t. injection of thrombin (1 pmol/mouse) or a recombinant human PDGF (0.1 pmol/mouse; PDGF) was performed in naive mice. I.t. pretreatment with either hirudin or PDGFRα/Fc was performed 30 min or 1 hr, respectively, before a single i.t. injection of thrombin.

Guanosine-5'-o-(3-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) binding assay

Seven days after nerve ligation or sham operation, for membrane preparation, spinal cords were rapidly excised at 4℃. The tissue was homogenized using a Potter-Elvejham tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris-HCl buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 1 mM EGTA. The homogenate was centrifuged at 4℃ for 10 min at 48,000×*g*. The pellet was resuspended in [^{35}S]GTPγS binding assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA and 100 mM NaCl and centrifuged at 4℃ for 10 min at 48,000×*g*. The final pellet was resuspended in [^{35}S]GTPγS binding assay buffer and stored at -70℃ until use. The membrane homogenate (3-8 μg protein/assay) was incubated at 25℃ for 2 hr in 1 ml of assay buffer with various concentrations of the agonist, 30 μM guanosine-5'-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was

terminated by filtration using a Brandel cell harvester (Model M-24, Brandel, MD, USA) and Whatman GF/B glass filters that had been presoaked in 50 mM Tris-HCl (pH 7.4), transferred to scintillation-counting vials containing 0.5 mL of a tissue solubilizer (Soluene-350, Packard Instrument Company, Meriden, CT, USA) and 4 mL of a scintillation cocktail (Hionic Fluor, Packard Instrument Company), and equilibrated for 12 hr, and the radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. The binding of [^{35}S]GTPγS was measured in the presence of GDP and the absence of agonist. The data are expressed as the mean±S.E.M. for percentage stimulation. Similar results were obtained from at least three independent sets of experiments. The membrane homogenate was incubated at 25℃ for 2 hr in 50 mM Tris-HCl buffer (pH 7.4) with 0.001-10 μM thrombin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a total volume of 1 mL. The reaction was terminated using a Brandel cell harvester (Model M-24, Brandel) and filtration through Whatman GF/B glass filters that had been presoaked in 50 mM Tris-HCl (pH 7.4), 0.01 % (w/v) polyethylenimine at 4℃ for 2 hr. Filters were washed three times with 5 mL of Tris-HCl buffer (pH 7.4, 4℃), and transferred to scintillation-counting vials containing 0.5 mL of tissue solubilizer (Soluene-350, Packard Instrument Company) and 4 mL of a scintillation cocktail (Hionic Fluor, Packard Instrument Company). After 12 hr, the radioactivity in the samples was determined with a liquid scintillation analyzer (Model 1600CA, Packard Instrument Company). The values from Scatchard plot analyses represent the mean±S.E.M. of three independent experiments.

Sample preparation

Seven days after nerve ligation or sham operation, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and intracardially perfusion-fixed with freshly prepared 4 % paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the lumbar spinal cord was quickly removed and post-fixed in 4 % paraformaldehyde for 2 hr, and permeated with 20 % sucrose in 0.1M PBS for 1 day and 30 % sucrose in 0.1M PBS for 2 days with agitation. The L5 lumbar spinal cord segments were then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at –30 ℃ until use. Frozen spinal cord segments were cut with a freezing cryostat (Leica CM 1510, Leica Microsystems AG, Wetzlar, Germany) at a thickness of 10 μm and thaw-mounted on poly-L-lysine-coated glass slides.

Immunohistochemistry

The spinal cord sections were blocked in 10 % normal goat serum (NGS) in 0.01M PBS for 1 hr at room temperature. Each primary antibody [1:50-150 thrombin R (PAR-1, Santa Cruz Biotechnology, Inc., CA, USA), 1:3000 protein kinase Cγ (PKCγ Santa Cruz Biotechnology, Inc.), 1:120 PDGF-A (Santa Cruz Biotechnology, Inc.), 1:400 neuronal nuclei (NeuN, Chemicon International Inc., Temecula, CA, USA), 1:1000 S100β-subunit clone SH-B1 (S100β, Sigma-Aldrich Co., St. Louis, MO, USA)] was diluted in 0.01 M PBS containing 10 % NGS and incubated for 2 nights at 4 °C.

The samples were then rinsed and incubated with an appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 for 2 hr at room temperature. Since the staining intensity might vary between experiments, control sections were included in each run of staining. The slides were then cover-slipped with PermaFluor Aqueous mounting medium (Immunon^{TM}; ThermoShandon, Pittsburgh, PA, USA). All sections were observed with a light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus).

RNA preparation and quantitative analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA in the spinal cord of thrombin-injected or PBS-injected mice was extracted using the SV Total RNA Isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. Purified total RNA was quantified spectro-photometrically at A_{260}. To prepare first-strand cDNA, 1 μg of RNA was incubated in 100 μL of buffer containing 10 mM dithiothreitol, 2.5 mM $MgCl_2$, dNTP mixture, 200 units of reverse transcriptase II (Life Technologies, Inc., USA) and 0.1 mM oligo $(dT)_{12\text{-}18}$ (Life Technologies, Inc.). The PDGF-A gene was amplified in a 50 μL PCR solution containing 0.8 mM $MgCl_2$, dNTP mixture and DNA polymerase with synthesized primers: a sense primer of PDGF-A, which is at position 407-423 (5'-CTGTGCCCATTCGCAGG-3') of the PDGF-A and an antisense primer at position 915-929 (5'-ACCGCACGCACATTG-3'), which was designed according to GenBank^{TM} sequence accession number AY324648. Samples were heated to 95 ℃

for 1 min, 55 ℃ for 2 min and 72 ℃ for 3 min. The final incubation was 72 ℃ for 7 min. The mixture was run on 2 % agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The agarose gel was stained with ethidium bromide and photographed with UV trans-illumination. The intensity of the bands was analyzed and semiquantified by computer-assisted densitometry using NIH image software. Values represent the mean±S.E.M. of three independent experiments.

Drugs

The drugs used in the present study were thrombin (Wako Pure Chemical Industries, Ltd.), a thrombin inhibitor hirudin (Sigma-Aldrich Co.), PDGFRα/Fc (R&D Systems, Inc., Minneapolis, MN, USA), a PDGF receptor-dependent tyrosine kinase inhibitor AG17 ([(3,5-di-tert-butyl-4-hydroxybenzylidene)-malononitrile]; Calbiochem-Novabiochem Co., La Jolla, CA, USA) and PDGF (R&D Systems, Inc.). Thrombin and PDGFRα/Fc were dissolved in sterile PBS (pH 7.4). Hirudin was dissolved in 0.9 % sterile physiological saline. AG17 was dissolved in 0.9 % sterile physiological saline containing 6 % dimethyl sulfoxide (DMSO). PDGF was dissolved in 0.1 % bovine serum albumin (BSA) containing 4 mM HCl.

Statistical analysis

All data are presented as the mean ± S.E.M. The statistical significance of differences between groups was assessed with two-way analysis of variance (ANOVA)

or one-way ANOVA followed by the Bonferroni/Dunn multiple comparison test or with Student's t-test.

Results

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation in mice were significantly suppressed by repeated i.t. injection of the thrombin inhibitor hirudin

Partial ligation of the sciatic nerve caused a marked decrease in the latency of paw withdrawal against a thermal stimulus only on the ipsilateral side in nerve-ligated mice (vs. Saline-Sham group, $F(1,7)=168.441$, $p<0.001$, Fig. 1-1A). The thermal hyperalgesia observed on the ipsilateral side in nerve-ligated mice was significantly suppressed by repeated i.t. injection of hirudin (30 pmol/mouse) just before ligation and once a day for 8 consecutive days after nerve ligation (vs. Saline-Ligation group, $F(1,9)=108.923$, $p<0.001$, Fig. 1-1A).

Mice with sciatic nerve ligation also showed a marked increase in paw withdrawal in response to a tactile stimulus only on the ipsilateral side in nerve-ligated mice (vs. Saline-Sham group, $F(1,7)=69.829$, $p<0.001$, Fig. 1-1B; vs. Saline-Sham group, $F(1,7)=124.623$, $p<0.001$, Fig. 1-1C). Under these conditions, repeated i.t. injection of hirudin (30 pmol/mouse) significantly suppressed the increase in paw withdrawal in response to an innocuous tactile stimulus induced by nerve ligation in mice (vs. Saline-Ligation group, $F(1,9)=40.565$, $p<0.001$, Fig. 1-1B; vs. Saline-Ligation group, $F(1,9)=37.352$, $p<0.001$, Fig. 1-1C). In addition, the recovery of the persistent reduction in the thermal threshold by hirudin lasted even after treatment with hirudin was terminated (from day 9 to day 15). (vs. Saline-Ligation group, $F(1,9)=34.934$,

$p<0.001$, Fig. 1B; vs. Saline-Ligation group, $F(1,9)=51.586$, $p<0.001$, Fig. 1-1C).

Repeated i.t. injection of hirudin at the doses used in the present study failed to affect thermal and tactile thresholds on the contralateral side in nerve-ligated mice and on both sides in sham-operated mice (data not shown).

[^{35}S]GTPγS binding by the PAR agonist thrombin to membranes of the spinal cord was significantly increased in nerve-ligated mice

The ability of the endogenous PAR ligand thrombin to activate G-proteins in the spinal cord of sham-operated or nerve-ligated mice was examined by monitoring the binding of [^{35}S]GTPγS to spinal cord membranes. Thrombin (0.001-10 μM) produced a concentration-dependent increase in the binding of [^{35}S]GTPγS to the L5 lumbar spinal cord membranes obtained from sham-operated mice. In membranes of the spinal cord from nerve-ligated mice, the increase in [^{35}S]GTPγS binding induced by thrombin was significantly greater than that obtained from sham-operated mice ($F(1,90)=141.7$, $p<0.001$, Fig. 1-2).

PAR-1-like IR in the dorsal horn of the mouse spinal cord was increased by sciatic nerve ligation

PAR-1-like immunoreactivity (IR) was detected on the ipsilateral side of the L5 lumbar spinal dorsal horn of sham-operated mice (Fig. 1-3A). Seven days after sciatic nerve ligation, PAR-1-like IR in lamina II and III of the ipsilateral side of the L5 lumbar spinal dorsal horn was increased compared to that observed in sham-operated mice (Fig.

1-3B). Furthermore, the increased PAR-1-like IR in the spinal cord after nerve injury was co-localized with PKCγ, which is highly limited to neuronal cells in the inner part of lamina II of the dorsal horn of the spinal cord (Fig. 1-4).

Thermal hyperalgesia and tactile allodynia induced by a single i.t. treatment with thrombin in normal mice were suppressed by i.t. pretreatment with hirudin

I next investigated whether exogenous i.t. treatment with thrombin could cause a hyperalgesic or allodynic response in normal mice. A single i.t. injection of thrombin (1 pmol/mouse) produced marked thermal hyperalgesia and tactile allodynia in normal mice after injection, and these effects lasted for 9-10 days after injection (vs. Saline-PBS group, $F(1,10)=605.881$, $p<0.001$, Fig. 1-5A; vs. Saline-PBS group, $F(1,10)=138.103$, $p<0.001$, Fig. 1-5B; vs. Saline-PBS group, $F(1,10)=247.557$, $p<0.001$, Fig. 1-5C). The long-lasting thermal hyperalgesia and tactile allodynia caused by an exogenous single i.t. injection of thrombin were abolished by i.t. pretreatment with the thrombin inhibitor hirudin (30 pmol/mouse) (vs. Saline-Thrombin group, $F(1,10)=818.014$, $p<0.001$, Fig. 1-5A; vs. Saline-Thrombin group, $F(1,10)=81.366$, $p<0.001$, Fig. 5B; vs. Saline-Thrombin group, $F(1,10)=111.593$, $p<0.001$, Fig. 1-5C).

PDGF-A-like IR was detected in the neuron and astrocytes in the mouse spinal cord

I investigated the localization of PDGF-A in the L5 lumbar spinal cord in mice using immunohistochemical analysis. PDGF-A-like IR was detected in the superficial

laminae and inner part of the L5 lumbar spinal cord. Furthermore, PDGF-A-like IR was principally co-localized with the neuron-specific nuclear protein marker NeuN-positive cells in the superficial and inner part of the dorsal horn. Astrocytes in the dorsal horn of the spinal cord were stained with S100β, a specific marker for astrocytes. PDGF-A-like IR was observed occasionally in S100β-positive cells in the dorsal horn of the spinal cord (Fig. 1-6).

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation in mice were suppressed by repeated i.t. injection of PDGFRα/Fc

Repeated i.t. injection of PDGFRα/Fc (10 ng/mouse) just before and once a day for 8 consecutive days after nerve ligation reversed the decreased thermal and tactile threshold on the ipsilateral side of nerve-ligated mice (vs. PBS-Ligation group, $F(1,10)=179.88$, $p<0.001$, Fig. 1-7A; vs. PBS-Ligation group, $F(1,10)=68.049$, $p<0.001$, Fig. 1-7B; vs. PBS-Ligation group, $F(1,10)=47.454$, $p<0.001$, Fig. 1-7C). The same treatment had no effect on the latency of paw withdrawal after a thermal or tactile stimulus on the contralateral side in nerve-ligated mice and on both sides in sham-operated mice (data not shown).

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly reversed by repeated i.t. injection of the PDGF receptor-dependent PTK inhibitor AG17

To investigate whether the activation of protein tyrosine kinase (PTK) mediated by

PDGF receptor could be directly involved in the neuropathic pain-like state induced by nerve ligation, groups of mice were treated i.t. with the PDGF receptor-dependent PTK inhibitor AG17 (30 nmol/mouse) just before surgery and once a day for 8 consecutive days after nerve ligation. The thermal hyperalgesia and tactile allodynia induced by nerve ligation were significantly reversed by chronic i.t. treatment with AG17 (vs. Vehicle-Ligation group, $F(1,11)=116.508$, $p<0.001$, Fig. 1-8A ; vs. Vehicle-Ligation group, $F(1,11)=20.928$, $p<0.001$, Fig. 1-8B ; vs. Vehicle-Ligation group, $F(1,11)=25.063$, $p<0.001$, Fig. 1-8C). Such treatment had no effect on the latency of paw withdrawal on the contralateral side in nerve-ligated mice or on both sides in sham-operated mice (data not shown).

Thermal hyperalgesia and tactile allodynia induced by a single i.t. treatment with PDGF in normal mice were suppressed by i.t. pretreatment with AG17

To investigate whether PDGF could cause a hyperalgesic or allodynic response in normal mice, groups of normal mice were treated i.t. with PDGF. A single i.t. injection of PDGF (0.1 pmol/mouse) produced marked thermal hyperalgesia and tactile allodynia in normal mice after injection, and this effect lasted for 8-9 days after injection (vs. Vehicle-PBS group, $F(1,8)=164.250$, $p<0.001$, Fig. 1-9A; vs. Vehicle-PBS group, $F(1,8)=197.951$, $p<0.001$, Fig. 1-9B; vs. Vehicle-PBS group, $F(1,8)=93.523$, $p<0.001$, Fig. 1-9C). The long-lasting thermal hyperalgesia and tactile allodynia caused by an exogenous single i.t. injection of PDGF were abolished by i.t. pretreatment with the PDGF receptor-dependent PTK inhibitor AG17 (30 nmol/mouse)

(vs. Vehicle-PDGF group, $F(1,8)=376.449$, $p<0.001$, Fig. 1-9A; vs. Vehicle-PDGF group, $F(1,8)=49.965$, $p<0.001$, Fig. 1-9B; vs. Vehicle-PDGF group, $F(1,8)=68.029$, $p<0.001$, Fig. 1-9C).

Thermal hyperalgesia and tactile allodynia induced by a single i.t. treatment with thrombin in normal mice were significantly eliminated by repeated i.t. pretreatment with PDGFRα/Fc

Facilitation of the activation of a thrombin/PAR-1/PDGF pathway in the spinal cord may play an important role in the development of the neuropathic pain-like state in mice. The next study was undertaken to investigate whether spinal PDGF could be involved in the thermal hyperalgesia or tactile allodynia observed with a single i.t. injection of thrombin. The thermal hyperalgesia and tactile allodynia induced by a single i.t. injection of thrombin were significantly eliminated by repeated i.t. pretreatment with PDGFRα/Fc just before injection and once a day for 12 consecutive days after injection (vs. PBS-Thrombin group, $F(1,9)=463.624$, $p<0.001$, Fig. 1-10A; vs. PBS-Thrombin group, $F(1,9)=38.222$, $p<0.001$, Fig. 1-10B; vs. PBS-Thrombin group, $F(1,9)=70.964$, $p<0.001$, Fig. 1-10C). Under these conditions, there were no differences between PBS- and thrombin-injected mice with regard to mRNA levels of PDGF-A in the spinal cord (Fig. 1-11).

PAR-1-positive cells were clearly co-localized with PDGF-A-like immunoreactivity in the dorsal horn of the spinal cord of nerve-ligated mice

The key approach in the present study was to investigate the co-localization of PAR-1 with PDGF-A in the dorsal horn of the spinal cord of nerve-ligated mouse. Interestingly, almost all of the PAR-1-positive cells in the dorsal horn of the spinal cord of nerve-ligated mice were clearly co-localized with PDGF-A-like IR, indicating that PDGF-A-like IR is highly located on PAR-1-positive neuronal cells (Fig. 1-12).

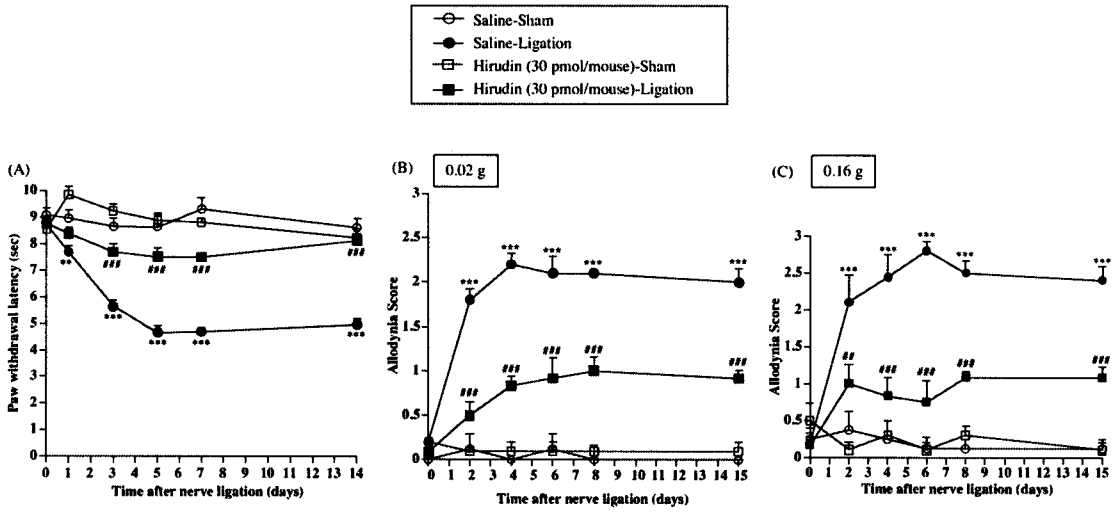


Fig. 1-1 Effect of repeated intrathecal (i.t.) injection of a thrombin inhibitor, hirudin, on thermal hyperalgesia (A) and tactile allodynia (B and C) in sham-operated or nerve-ligated mice. Tactile stimulus was applied using filaments with two different bending forces [0.02 g (B) and 0.16 g (C)]. Groups of mice were repeatedly treated i.t. with hirudin (30 pmol /mouse) or saline 30 min prior to surgery (day 0) and once a day for 8 consecutive days after surgery. From day 9 to day 15 after surgery, mice were not treated with hirudin. Each point represents the mean ± S.E.M. of 5-7 mice. **p<0.01 and ***p<0.001 vs. Saline-Sham group, ##p<0.01 and ###p<0.001 vs. Saline-Ligation group.

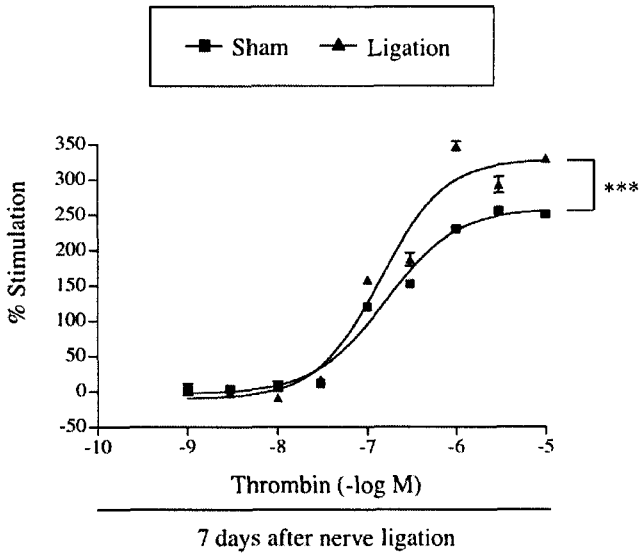


Fig. 1-2 Comparison of the stimulation of [^{35}S]GTPγS binding by the PAR agonist thrombin to membranes of the spinal cord obtained from sham-operated or nerve-ligated mice. Sample preparation was performed at 7 days after nerve ligation. The spinal cord membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with or without thrombin. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and the absence of thrombin. Values represent the mean ± S.E.M. of 3 samples. $F(1, 90)=141.7$, $p<0.001$ vs. Sham group.

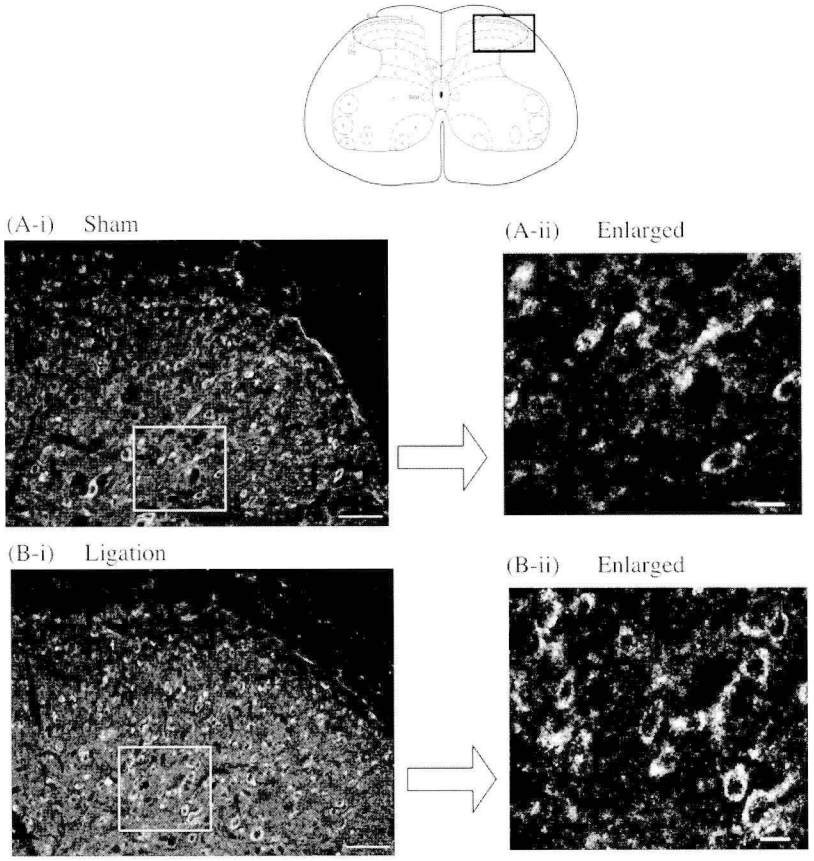


Fig. 1-3 Increase in PAR-1-like immunoreactivity (IR) on the ipsilateral superficial dorsal horn of the L5 lumbar spinal cord in nerve-ligated mice (B-i and B-ii) compared to that in sham-operated mice (A-i and A-ii). Spinal cord slices were prepared 7 days after sham operation or nerve ligation in mice. Scale bars; 50 μm (A-i and B-i), 10 μm (A-ii and B-ii).

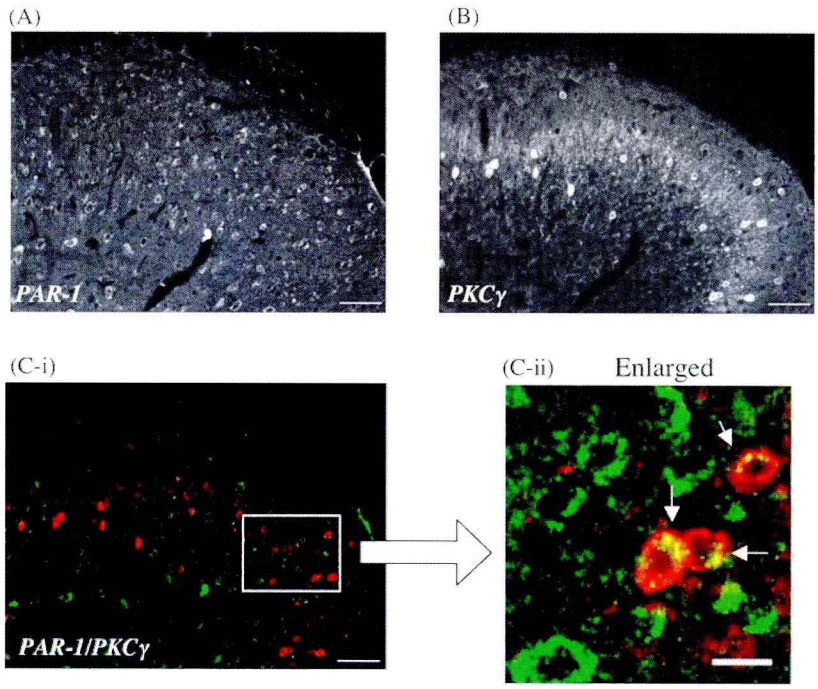


Fig. 1-4 Co-localization of PAR-1 (A) with PKCγ, which is highly limited to the inner part of lamina II in the dorsal horn of the mouse spinal cord (B). The green labeling for PAR-1 and the red labeling for PKCγ show co-localization in the dorsal horn of the spinal cord (C-i, -ii). PAR-1-like IR is seen in the membrane of PKCγ-labelled cells in the dorsal horn of the spinal cord (arrow head in C-ii). Scale bars=50 μm (A, B and C-i), 10 μm (C-ii).

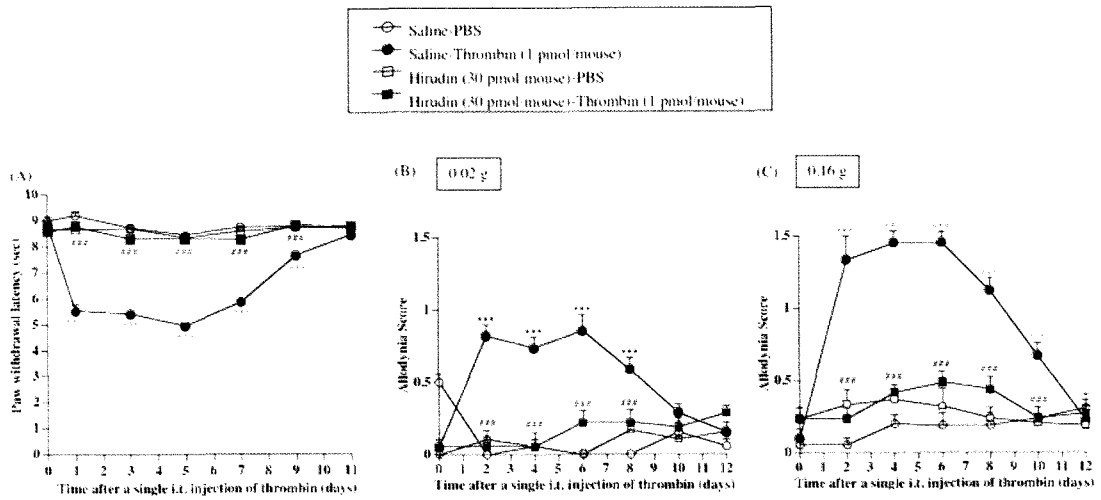


Fig. 1-5 Effect of a single i.t. injection of thrombin on paw withdrawal responses to the thermal (A) and tactile (B and C) stimulus in normal mine. Tactile stimulus was performed using filament with two different bending forces [0.02 g (B) and 0.16 g (C)]. Group of mice were repeatedly treated i.t. with a thrombin inhibitor hirudin (30 pmol/mouse) or saline 30 min before a single i.t. injection of thrombin and once a day for 12 consecutive days after injection. Each point represents the mean ± S.E.M. of 6 mice. ***p<0.001 vs. Saline-PBS group, ##p<0.01 and ###p<0.001 vs. Saline-Thrombin group.

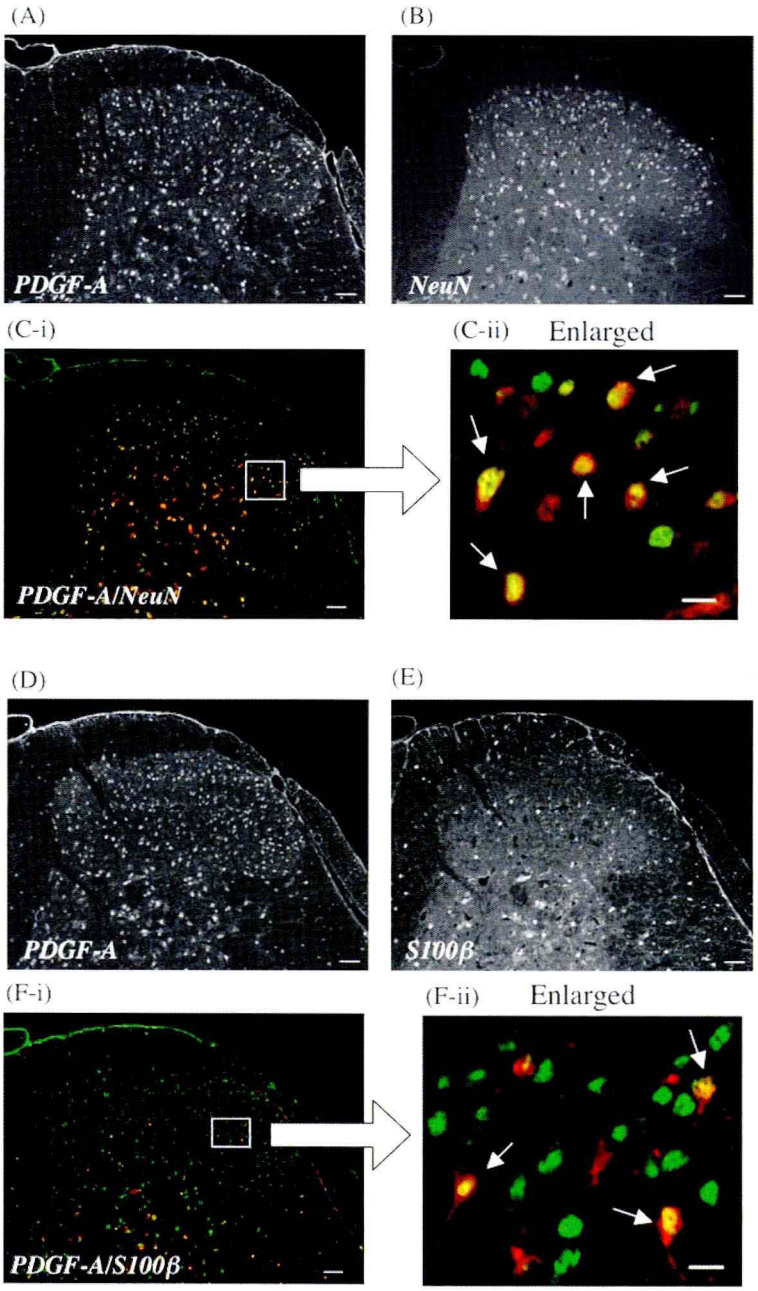


Fig. 1-6 Co-localization of PDGF-A (A, D) with NeuN, a neuron-specific nuclear protein marker, (B) or S100β, a specific marker for astrocytes, (E) in the dorsal horn of the mouse spinal cord. The green labeling for PDGF-A and the red labeling for NeuN or S100β show co-localization in the dorsal horn of the spinal cord (C-i, -ii or F-i, -ii). PDGF-A-like IR is exclusively observed on NeuN-labelled cells (C-ii) and partially observed on S100β-labelled cells in the dorsal horn of the spinal cord (F-ii). Scale bars=50 μm (A, B, C-i, D, E and F-i), 10 μm (C-ii and F-ii).

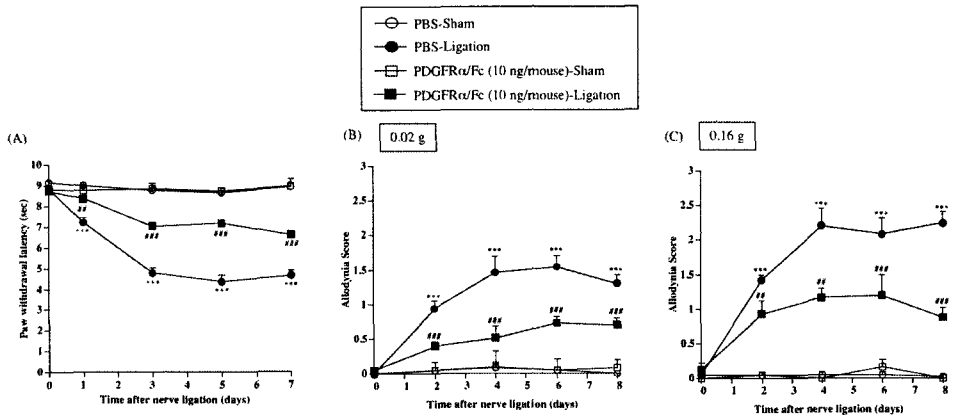


Fig. 1-7 Effect of repeated i.t. injection of PDGFRα/Fc on thermal hyperalgesia (A) and tactile allodynia (B and C) in sham-operated or nerve-ligated mice. Tactile stimulus was applied using filaments with two different bending forces [0.02 g (B) and 0.16 g (C)]. Groups of mice were repeatedly treated i.t. with PDGFRα/Fc (10 ng/mouse) or sterile PBS 1 hr prior to surgery (day 0) and once a day for 8 consecutive days after surgery. Each point represents the mean ± S.E.M. of 5-6 mice. ***p<0.001 vs. PBS-Sham group, ##p<0.01 and ###p<0.001 vs. PBS-Ligation group.

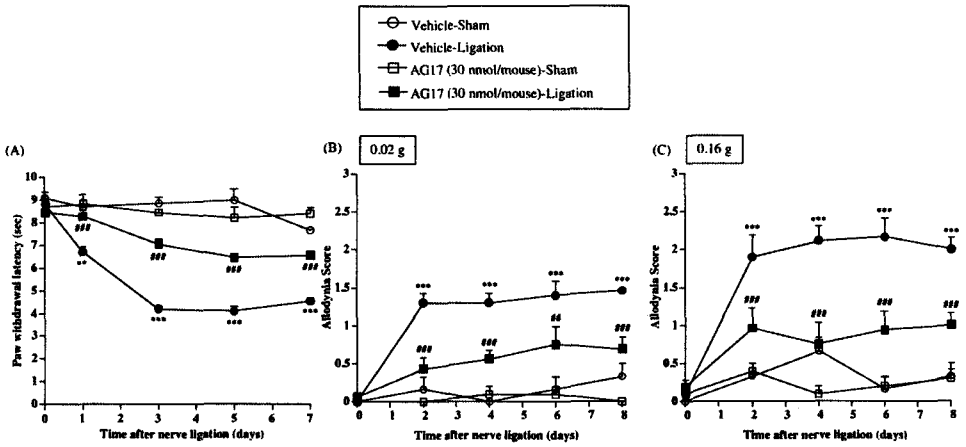


Fig. 1-8 Effect of repeated i.t. injection of an inhibitor of PDGF receptor-mediated PTK, AG17, on thermal hyperalgesia (A) and tactile allodynia (B and C) in sham-operated or nerve-ligated mice. Tactile stimulus was applied using filaments with two different bending forces [0.02 g (B) and 0.16 g (C)]. Groups of mice were repeatedly treated i.t. with AG17 (30 nmol/mouse) or vehicle 30 min prior to surgery (day 0) and once a day for 8 consecutive days after surgery. Each point represents the mean ± S.E.M. of 5-7 mice. **p<0.01 and ***p<0.001 vs. Vehicle-Sham group, ##p<0.01 and ###p<0.001 vs. Vehicle-Ligation group.

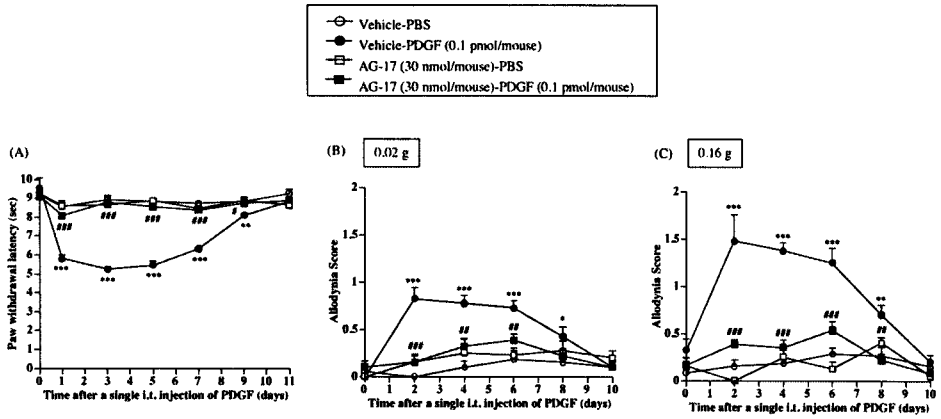


Fig. 1-9 Effect of a single i.t. injection of PDGF on paw withdrawal responses to the thermal (A) and tactile (B and C) stimulus in normal mine. Tactile stimulus was performed using filament with two different bending forces [0.02 g (B) and 0.16 g (C)]. Group of mice were repeatedly treated i.t. with an inhibitor of PDGF receptor-mediated PTK, AG17 (30 nmol/mouse) or vehicle 30 min before a single i.t. injection of PDGF and once a day for 11 consecutive days after injection. Each point represents the mean ± S.E.M. of 6 mice. *p<0.05, **p<0.01 and ***p<0.001 vs. Vehicle-PBS group, #p<0.05, ##p<0.01 and ###p<0.001 vs. Vehicle-PDGF group.

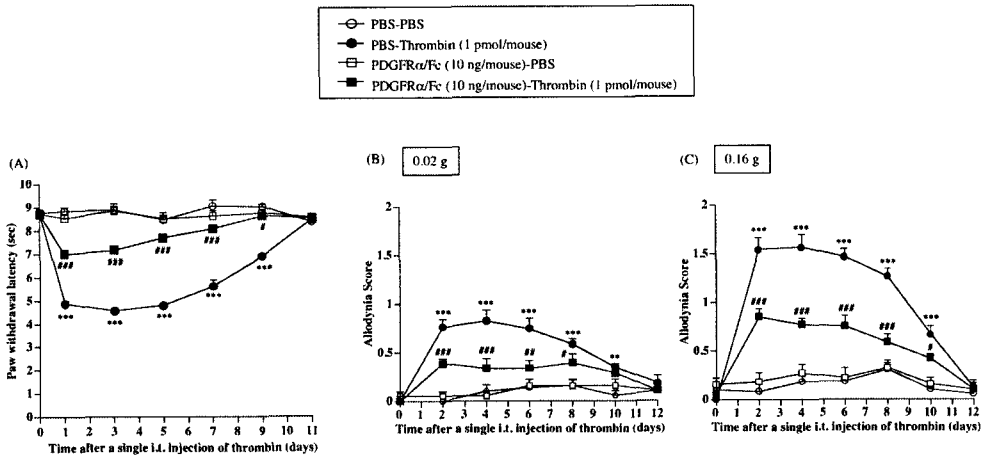


Fig. 1-10 Effect of repeated i.t. injection of PDGFRα/Fc on thermal hyperalgesia or tactile allodynia induced by a single i.t. injection of thrombin in normal mice. Groups of mice were repeatedly treated i.t. with PDGFRα/Fc (10 ng/mouse) or sterile PBS 1 hr prior to the single i.t. injection of thrombin (1 pmol/mouse) and once a day for 12 consecutive days after injection. Each point indicates the mean ± S.E.M. of 6 mice. **p<0.01 and ***p<0.001 vs. PBS-PBS group, #p<0.05, ##p<0.01 and ###p<0.001 vs. PBS-Thrombin group.

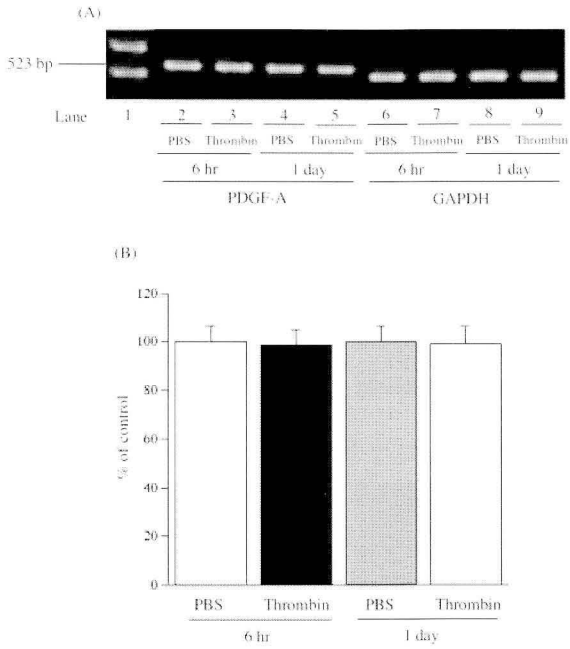


Fig. 1-11 Representative RT-PCR for PDGF-A (lanes 2-5) and an internal standard GAPDH (lanes 6-9) (A) mRNAs in the spinal cord at 6 hr and 1 day after a single i.t. injection of PBS (lanes 2, 4, 6, 8) or thrombin (1 pmol/mouse) (lanes 3, 5, 7, 9) in mice. Lane 1 is the indicated marker. The intensity of the bands was determined in a semi-quantitative manner using NIH Image (B). Each value for PDGF-A mRNA in thrombin-injected mice was normalized by the value for the respective GAPDH mRNA. The values in thrombin-injected mice are expressed as a percentage of the increase in PBS-injected mice. Each column represents the mean ± S.E.M. of 10 samples.

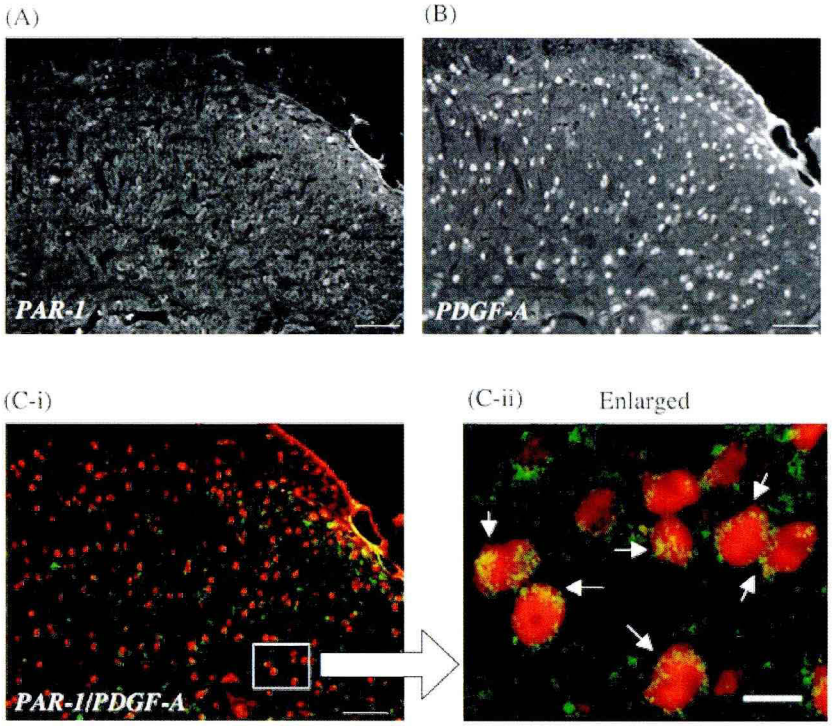


Fig. 1-12 Co-localization of PAR-1 (A) with PDGF-A (B) in the dorsal horn of the mouse spinal cord. The green labeling for PAR-1 and the red labeling for PDGF-A show apparent co-localization in the dorsal horn of the spinal cord (C-i, -ii). PDGF-A-like IR is almost all seen in the cytoplasm of PAR-1-labeled cells in the dorsal horn of the spinal cord (arrow head in C-ii). Scale bars=50 μm (A, B and C-i), 10 μm (C-ii).

Discussion

A very large number of studies have confirmed that a serine protease, thrombin, is a major stimulus for platelets, and initiates a series of co-ordinated events that result in platelet aggregation *in vitro* or *in vivo* [75]. In addition to its well-known role in the platelet aggregation cascade, thrombin has been implicated in degenerative as well as protective mechanisms in the CNS. It is of interest to note that PAR-1, one of the thrombin receptor subtypes, is expressed in a subset of small and medium diameter of primary sensory neurons, suggesting that a thrombin/PAR-1 pathway play a possible role in the transmission of pain [80]. It has been reported that responses to thrombin were found to be blocked by the specific thrombin inhibitor hirudin, a hirudin-derived peptide, which reflects the thrombin-specific nature of the cloned receptor. Several studies have demonstrated that the response to thrombin is unrelated to the proteolytic properties of the enzyme, but rather the hirudin-like binding domain appears to be the important feature of the protein. Mutation of the N-terminus identified the presence of a hirudin-like domain that was essential for high-affinity binding and the potent effects of thrombin [75]. The hirudin-like thrombin binding sequence has been identified in PAR-1. This would perhaps allow high-affinity binding to PAR-1 in the hirudin-like binding domain. In the present study, I first investigated the role of a spinal thrombin/PAR-1 pathway in the development of a neuropathic pain-like state in mice. Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly suppressed by i.t. pretreatment with hirudin just before and once a day for

the first 7 days after surgery. Interestingly, the recovery of the persistent reduction in the thermal threshold by hirudin lasted even after treatment with hirudin was discontinued, which indicates the importance of the initial blockade of PAR-1 for inhibiting the development of a neuropathic pain-like state. In the preliminary study, I also investigated whether the thrombin/PAR-1 pathway within the spinal cord could be involved in the maintenance, as well as the development, of a neuropathic pain-like state in mice. The developed neuropathic pain-like state was significantly suppressed even by repeated i.t. post-treatment with hirudin, which was started from 7 days after sciatic nerve ligation (thermal hyperalgesia; 66.4±2.68 % of maximum inhibition, tactile allodynia; 82.5±7.85 % of maximum inhibition). These results suggest that the thrombin/PAR-1 pathway within the spinal cord may also contribute to the maintenance of a neuropathic pain-like state. Furthermore, I demonstrated here that an exogenous single i.t. injection of thrombin produced long-lasting thermal hyperalgesia and tactile allodynia in normal mice. These responses were abolished by i.t. pretreatment with hirudin. These findings indicate that the spinal thrombin/PAR-1 pathway may be directly involved in the development of a neuropathic pain-like state caused by nerve ligation in mice.

Additional evidence for the contribution of a spinal thrombin/PAR-1 pathway to a neuropathic pain-like state was obtained by an [^{35}S]GTPγS binding assay and immunohistochemical approach. Possible changes in PAR-1 function in the spinal cord of nerve-ligated mice were evaluated by monitoring the binding of [^{35}S]GTPγS to membranes of the mouse spinal cord. In membranes of the L5 lumbar spinal cord

from nerve-ligated mice, the concentration-dependence curve for the increased [^{35}S]GTPγS binding induced by thrombin was significantly greater than that obtained in sham-operated mice. Furthermore, an immunohistochemical study showed that sciatic nerve ligation produced a clear increase in PAR-1-like IR on lamina II and III of the ipsilateral side of the L5 lumbar spinal dorsal horn compared to that observed in sham-operated mice. The subpopulations of PAR-1-like IR in the spinal cord were co-labelled with PKCγ-like IR, which is highly limited to neuronal cells in the inner part of lamina II of the dorsal horn of the spinal cord, indicating that PAR-1 in the dorsal horn of the L5 lumbar spinal cord may be mostly located in neurons. These findings provide further evidence that a thrombin/PAR-1 pathway within neuronal cells of the L5 lumbar spinal cord plays a critical role in the development of a neuropathic pain-like state.

PDGF was first identified in a search for serum factors that stimulate the proliferation of arterial smooth muscle cells [88]. Although the α-granule of platelets is a major storage site for PDGF, recent studies have shown that PDGF can be synthesized by several different cell types [86]. In the present study, PDGF-A-like IR was detected in the superficial laminae and inner part of the L5 lumbar spinal cord in mice. Furthermore, PDGF-A-like IR in the spinal cord was predominantly located on neuronal cells and occasionally located on astrocytes. Under these conditions, I found here for the first time that thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly suppressed by repeated i.t. treatment with PDGFRα/Fc, which can trap respective endogenous PDGF, resulting in the prevention of receptor

activation. Furthermore, a single i.t. injection of PDGF produced marked thermal hyperalgesia and tactile allodynia in normal mice after injection, and these effects lasted for 8-9 days after injection. These responses were abolished by i.t. pretreatment with a potent inhibitor of PTK activity linked to PDGF receptors, AG17. The reduction in the latency of paw withdrawal on the ipsilateral side in nerve-ligated mice was abolished by repeated i.t. injection of AG17. Considering these findings, the present data suggest that the release of PDGF within the spinal cord by nerve ligation may lead to the development of a neuropathic pain-like state in mice.

Recently, thrombin has been shown to activate platelets and promote the expression or the release of PDGF from platelets. It has also been suggested that the activation of thrombin receptor leads to several intracellular signaling events as well as to the stimulation of endogenous PDGF-A production [83]. In the present study, I demonstrated here that PDGF-A-like IR was detected in the neuronal cells and astrocytes within the L5 lumbar spinal cord. It is of interest to note that PDGF-A-like IR was co-localized with PAR-1 in the dorsal horn of the L5 lumbar spinal cord. Taken together, these findings raise the fascinating possibility that the activation of PAR-1 by thrombin may facilitate the expression or the release of PDGF in neuronal cells and astrocytes located in the spinal cord.

The next study was then undertaken to investigate whether spinal PDGF could be involved in thermal hyperalgesia or tactile allodynia mediated through the thrombin/PAR-1 pathway. The thermal hyperalgesia and tactile allodynia induced by a single i.t. injection of thrombin were significantly eliminated by repeated i.t.

pretreatment with PDGFRα/Fc just before injection and once a day for 9 consecutive days after injection. Under these conditions, there were no differences between PBS- and thrombin-injected mice with regard to mRNA levels of PDGF-A in the spinal cord. These results suggest that thrombin may activate PDGF-A-containing fibers in the spinal cord without changing the expression of PDGF-A mRNAs, resulting in the induction of hyperalgesia.

In a subsequent study, I investigated the co-localization of PAR-1-containing cells with PDGF-A in the dorsal horn of the spinal cord of nerve-ligated mice. Interestingly, almost all of the PAR-1-like IR-positive cells in the dorsal horn of the spinal cord of nerve-ligated mice were clearly co-labelled with PDGF-A-like IR, indicating that PDGF-A is mostly located on PAR-1-containing neuronal cells.

Released PDGF acts on PDGF receptor. It is well recognized that the activation of PDGF receptor and its autophosphorylation serve as docking sites for adapter proteins and enzymes such as phosphoinositide-3 kinase (PI-3 kinase), phospholipase C (PLC)γ and the Src family of tyrosine kinases [86]. Especially, the activation of PLCγ triggers the facilitation of PKC, which involves in the development of a neuropathic pain-like state [89,90], through the production of diacylglycerol and inositol 1,4,5-triphosphate (IP_3). Furthermore, BDNF, which was observed almost entirely on pre-synaptic neurons of the L5 lumbar mouse spinal cord, is an important modulator of the expression of neurotrophin associated with the activation of PKC [91,92]. However, I found here that PDGF-A-positive cells are predominantly located on post-synaptic neurons and are widely expressed in the outer and inner parts of the laminae of the spinal cord in mice.

These findings suggest that spinal PDGF may be more essential for the development of the neuropathic pain-like state induced by nerve ligation in mice. Although I clearly show here that both PAR-1 and PDGF are responsible for this type of the neuropathic pain-like state, I can not completely exclude the possibility that these two components are just necessary for the present model to be developed. Thus, farther investigation is needed to identify the role of PAR-1 and PDGF in other types of chronic pain models.

In conclusion, the present data provide novel evidence that PAR-1/PKCγ /PDGF-A-mediated signaling pathway within the spinal cord are directly involved in the development of the neuropathic pain-like state induced by sciatic nerve ligation in mice. Such findings raise the fascinating possibility that the activation of up-regulated PAR-1 located on the spinal dorsal horn neurons following nerve injury may cause a PKCγ-dependent PDGF release in the spinal cord and in turn activate PDGFRα, leading to a neuropathic pain-like state.

Chapter 2

Implication of spinal protein kinase Cγ isoform in activation of the mouse brain by intrathecal injection of the protein kinase C activator phorbol 12,13-dibutyrate using functional magnetic resonance imaging analysis

Introduction

The PKC family of enzymes plays an important role in signal transduction in several physiological processes [93]. The PKC family consists of at least 12 isoforms that differ with regard to their structure, substrate requirements, expression and localization, and therefore may underlie diverse physiological functions [94,95]. A growing body of evidence suggests that the activation of PKC in the spinal cord is implicated in changes in pain perception [89,96]. Interestingly, mice that lack the PKCγ isozyme show normal responses to acute pain stimuli but show reduced signs of neuropathic pain after partial ligation of the sciatic nerve, which suggests that the PKCγ isozyme is involved in the processing of nociception in the spinal cord [30]. This contention can be supported by the finding described in Chapter 1 that PKCγ is a key player to modulate a neuropathic pain-like state following sciatic nerve ligation in mice. It has been also reported that a single i.t. injection of a specific PKC activator, phorbol 12,13-dibutyrate (PDBu), produced long-lasting thermal hyperalgesia and pain-like behavior as indicated by severe tail-shaking, vocalization, scratching and biting behaviors in mice [27,28].

Functional magnetic resonance imaging (fMRI) can be used to investigate spatial and temporal brain activation evoked by physiological and pharmacological interventions. The benefits of this approach over conventional ex vivo methods include its non-invasive nature and the ability to monitor rapid temporal changes in brain activity at high anatomical resolution [97]. fMRI has been used to evaluate central nervous system processing of experimental pain in humans [39,98]. Noxious heat

stimulation in humans activates several brain regions, including the thalamus, basal ganglia, insula, cingulate cortex, somatosensory, premotor and motor cortices, prefrontal and inferior parietal cortex [99-102]. Animal studies on spinal cord and brain activation following noxious stimulation have used autoradiography with 2-deoxyglucose and the radiotracer [^{99m}Tc] exametazime, and more recently fMRI. Recent fMRI studies in rat models of pain have focused on activation of the cortical area and thalamus, as determined by the blood oxygen level-dependent (BOLD) contrast method, following noxious stimulation with the intraplantar injection of formalin or capsaicin [103,104]. However, the effects of neuropathic pain-like transmission evoked by the activation of spinal PKC on the BOLD response, particularly with regard to the relationship between the spatial profile and the time-course of activation, are unknown.

The purpose of this study was to investigate whether direct spinal PKC activation could cause a change in brain activation using the fMRI method in mice with a deletion of the gene that encodes the neuron-specific PKCγ isoform.

Materials and Methods

Animals

PKCγ knockout mice and their wild-type mice were used in the present study. PKCγ knockout mice were maintained on mixed genetic background of C57BL/6J and 129Sv (The Jackson Laboratory, Bar Harbor, ME). Animals were housed in a room maintained at 23 ± 1 °C with a 12 hr light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water were available *ad libitum.*

Intrathecal (i.t.) injection

Intrathecal (i.t.) injection was performed following the method described in Chapter 1.

Functional imaging

Mice were anaesthetized using isoflurane immediately after i.t. injection of PDBu or vehicle. Animals were then transferred to a cradle designed to fit inside the probe of the MR system and supplied with 1% isoflurane via a fitted mask. A continuous fMRI scanning protocol was used to study changes in brain signal intensity using T2-weighted BOLD contrast, and the intrathecal injection of PDBu (Sigma-Aldrich Co., MO, USA). BOLD responses were measured hourly from 1 hr to 6 hr at all brain levels.

Experiments were performed with a Unity Inova spectrometer (Varian, Palo Alto, CA) which was interfaced to a 9.4-T/31-cm horizontal bore magnet equipped with

actively shielded gradients capable of 300 mT/m in a risetime of 500 sec (Magnex Scientific, Abingdon, UK). High-resolution anatomical scans were collected using a fast spin echo pulse sequence (TR = 2000 ms, TE = 45 ms, FOV = 25.6 x 25.6 mm^2, 1.0 mm slice thickness, 256 x 256 data matrix)

Functional images were obtained with a multi-slice gradient-echo fast imaging sequence (TE = 25 msec, TR = 70 msec, 30° flip angle, 128 x 128). One-mm-thick slices were acquired simultaneously over a field of view (FOV) of 25.6 mm^2 with an acquisition time of approximately 32 sec. Typically, 3-6 images were collected at baseline, followed by PDBu injection.

Regions of interest (ROIs) were selected and statistical analyses were performed using the image-analysis software ImageJ (Wayne Rasband, http://rsb.info.nih.gov/ij/; Karl Schmidt http://www.quickvol.com). ROIs were drawn according to an atlas of the mouse brain [105]. BOLD signal intensity values in each ROIs were extracted and normalized to the time of baseline (expressed as a percent change from baseline). Statistical analysis was performed to compare percent changes in BOLD signal intensity and activated pixels between baseline and each time point after PDBu injection. Pixels whose BOLD percentage change relative to the baseline period was significantly different at a 95% confidence level were overlaid onto the reference anatomical data set.

Drug

The drug used in the present study was a specific PKC activator PDBu (Sigma Co., St Louis, Mo., USA). PDBu was dissolved in 10% dimethyl sulfoxide (DMSO) containing 0.9% saline.

Statistical data analysis

The data are presented as the mean ± SEM. The statistical significance of differences between groups was assessed by one- or two-way analysis of variance (ANOVA) followed by the Bonferroni/Dunnett test or by Student's *t*-test.

Result

I investigated the changes in BOLD signal intensity in several brain regions following the direct activation of spinal PKC in mice. As shown in Fig.13, i.t. injection of PDBu caused a robust positive signal activity in several brain regions of wild-type mice compared with the basal intensity. Although the signal intensity in these regions was significantly increased, notable differences among these regions were seen in the time-course and levels of activation. The somatosensory cortex, lateral thalamic nuclei and medial thalamic nuclei, which are sensory-discriminative components of pain, were clearly activated following i.t. injection of PDBu in wild-type mice (Fig.2A – 2C). In the somatosensory cortex, i.t. injection of PDBu produced a dramatic and time-dependent increase in signal intensity at 1-6 hr after the injection (Fig. 2A-iii, *p<0.05, **p<0.01, ***p<0.001 vs. basal intensity; vehicle in wild-type vs. PDBu in wild-type, $F_{1,123}$=8.449, P<0.0001). The thalamic areas were activated with relatively moderate increases in signal intensity by the i.t. injection of PDBu at 1-6 hr in wild-type mice (medial thalamus: Fig. 2B-iii, *p<0.05, **p<0.01 vs. basal intensity; vehicle in wild-type vs. PDBu in wild-type, $F_{1,128}$=3.094, P<0.0001. lateral thalamus: Fig. 2C-iii, **p<0.01 vs. basal intensity; vehicle in wild-type vs. PDBu in wild-type, $F_{1,128}$=5.104, P<0.0001). These effects were abolished in mice which lacked the PKCγ gene (Fig. 2A-iii – 2C-iii).

The cingulate cortex, nucleus accumbens and ventral tegmental area, which are affective-motivational components of pain, including the mesolimbic dopaminergic

system, were also activated following i.t. injection of PDBu in wild-type mice (Fig. 2D – 2F). In the cingulate cortex, i.t. injection of PDBu produced a robust and significant increase in signal intensity at 3-6 hr (Fig. 2D-iii, *p<0.05, **p<0.01 vs. basal intensity; vehicle in wild-type vs. PDBu in wild-type, $F_{1,84}$=2.341, P<0.0001), but not at 1-2 hr. Activation of the mesolimbic areas that contain the nucleus accumbens and ventral tegmental area was noted with a only slight, but still significant, increase in signal intensity by i.t. injection of PDBu in wild-type mice (Fig. 2E – 2F). An i.t. injection of PDBu increased the signal intensity at 4-6 hr in the nucleus accumbens (Fig. 2E-iii, **p<0.01 vs. basal intensity; vehicle in wild-type vs. PDBu in wild-type, $F_{1,111}$=2.425, P<0.0001) and at 3-6 hr in the ventral tegmental area (Fig. 2F-iii, *p<0.05, **p<0.01 vs. basal intensity; vehicle in wild-type vs. PDBu in wild-type, $F_{1,95}$=1.380, P<0.0001). These effects were eliminated in mice which lacked the PKCγ gene (Fig. 2D-iii – 2F-iii). Under these conditions, control mice that had been injected i.t. with vehicle (10% DMSO in saline) did not show activation in any regions in mice of both genotypes (Fig. 2A-iii – 2F-iii).

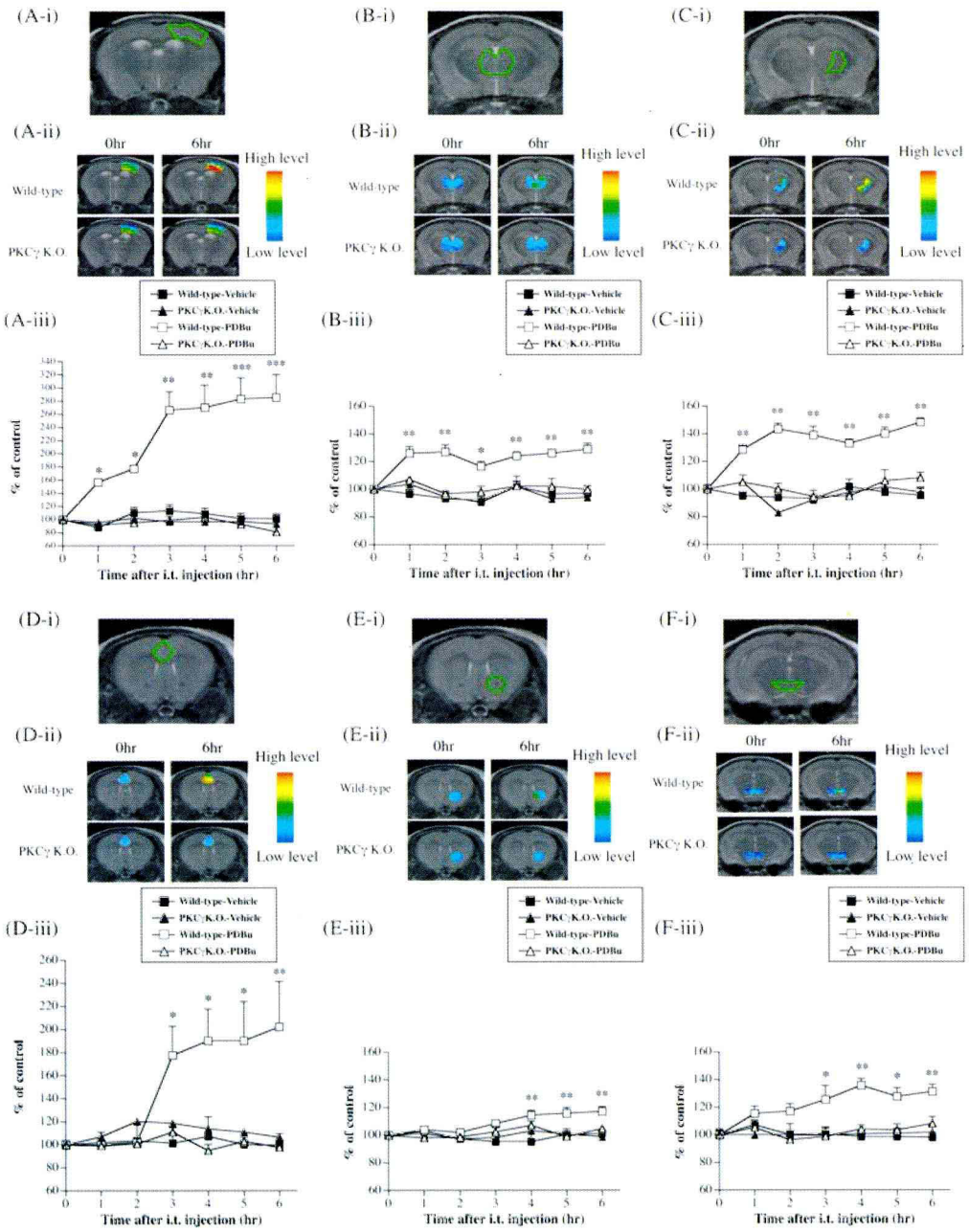


Fig. 2

Time-course of the effect of i.t. injection of PDBu on BOLD signal intensity in several brain regions in wild-type and PKCγ knockout mice. Intrathecal injection of PDBu produced a significant increase in BOLD signal compared with vehicle in wild-type mice. Representative high-resolution anatomical image shows regions of interest of the somatosensory cortex (A-i), lateral thalamus (B-i), medial thalamus (C-i), cingulate cortex (D-i), nucleus accumbens (E-i) and ventral tegmental area (F-i) in a coronal section. Representative activation maps (overlaid on anatomy) correspond to composite images of 0 hr or 6 hr after i.t. injection of PDBu in wild-type mice and PKCγ knockout mice (A-ii : somatosensory cortex, B-ii : lateral thalamus, C-ii : medial thalamus, D-ii : cingulate cortex, E-ii : nucleus accumbens, F-ii : ventral tegmental area). Intrathecal injection of PDBu produced a significant increase in BOLD signal compared with vehicle in wild-type mice (A-iii : somatosensory cortex, B-iii : the lateral thalamus, C-iii : medial thalamus, D-iii : cingulate cortex, E-iii : nucleus accumbens, F-iii : ventral tegmental area). Each point indicates the mean ± S.E.M. of 6-15 images. *$p<0.05$, **$p<0.01$, ***$p<0.001$, each post i.t. PDBu treatment intensity vs. basal intensity.

Discussion

It has been reported that PDBu as a phorbol ester binds to all PKC isotypes excluding aPKCs [106]. It is well recognized that the up-regulation of PKC activity in the spinal cord is a key factor for the induction of thermal hyperalgesia following nerve ligation. It has been reported that a single i.t. injection of PDBu produced a persistent pain-like state [27,28]. Furthermore, I confirmed an increase in the phosphorylation of all three subgroups (classic-type, novel-type and atypical-type) of PKC in the dorsal horn of spinal cord in mice following sciatic nerve ligation (data not shown). It is considered to be worthwhile to investigate ascending nociceptive transmission from the dorsal horn of spinal cord areas to brain areas involved in the processing of noxious stimuli induced by the activation of spinal PKCs. In the present study, I found that i.t. injection of PDBu caused a robust positive signal activity in several brain regions of wild-type mice compared with basal intensity. In contrast, these effects were eliminated in PKCγ gene knockout mice. These results suggest that the activation of several brain regions caused by spinal PKC activation is substantially dependent on spinal PKCγ isoform activation.

Another key finding of the present study was that there were notable differences in brain activation by the i.t. injection of PDBu among brain regions with regard to the time-course and level of activation. In the somatosensory cortex and lateral and medial thalamic nuclei of wild-type mice, i.t. injection of PDBu produced a significant increase in signal activity at 1-6 hr after the injection compared with basal intensity.

These effects were abolished in PKCγ gene knockout mice. In contrast, i.t. injection of PDBu induced a significant increase in signal intensity at 3-6 hr in the cingulate cortex, at 4-6 hr in the nucleus accumbens and at 3-6 hr in the ventral tegmental area. These results suggest that delayed activation can be clearly noted in the affective-motivational components of pain in i.t. PDBu-injected wild-type mice.

In conclusion, my analysis of PKCγ knockout mice supports the notion that loss of the PKCγ gene prevents the transmission of nociceptive information from the dorsal horn of the spinal cord to brain areas involved in the processing of noxious stimuli following i.t. injection of PDBu. I propose here that the activation of spinal PKCγ associated with the activation of ascending pain transmission may be an important factor in the development of a neuropathic pain-like state linked to emotional dysfunction.

Chapter 3

Implication of endogenous β-endorphin in the inhibition of the morphine-induced rewarding effect by the direct activation of spinal protein kinase C in mice

Introduction

Several clinical studies have suggested that in only a few cases can psychological dependence on opioids be considered to be a serious side-effect, when patients suffer from severe pain. Previously, it has been demonstrated that morphine failed to induce a rewarding effect in rodents under a chronic pain-like state [63,67,107]. The mesolimbic dopaminergic system, which projects from the VTA of the midbrain to the N.Acc., has been identified as the critical substrate of the rewarding effect of morphine [108,109]. It has been reported that inflammatory nociception activated endogenous κ-opioidergic systems to decrease dopamine release in the brain, resulting in suppression of the morphine-induced rewarding effect [67]. In contrast, sciatic nerve ligation suppressed morphine-induced dopamine release in the N.Acc., and this was associated with the inhibition of μ-opioid receptor-mediated G-protein activation in the VTA [65]. These findings strongly support the idea that a chronic pain-like state may lead to a change in the endogenous opioidergic system linked to the reward circuit. Interestingly, a s.c. morphine-induced place preference was significantly suppressed by a single i.t. pretreatment with PDBu [28]. Accordingly, activation of PKC in the spinal cord contributes to the attenuation of the morphine-induced rewarding effect through an increase in the sensitivity of spinal cord neuronal responses to excitatory inputs. In Chapter 2, I performed that activation of spinal PKCγ lead to the activation of ascending pain transmission, resulting in the increase in the activity of several brain regions including mesolimbic dopaminergic system.

Therefore, I hypothesized that activation of PKC in the spinal cord could lead to changes in the endogenous opioidergic system, thereby contributing to attenuation of the morphine-induced rewarding effect. The aim of present study was to investigate the role of an endogenous μ-opioid peptide β-endorphin in hyperalgesia and suppression of the morphine-induced rewarding effect by the PDBu-induced activation of PKC in the spinal cord using β-endorphin knockout mice.

Materials and methods

Animals

In the present Chapter, I used the male and female β-endorphin peptides derived from *propiomelanocortin (Pomc)* gene-knockout mice (The Jackson Laboratory, Bar Harbor, ME, USA), which were C57BL/6J and 129S2/SvPas mixed genetic background as described previously by Rubinstein et al. [110]. Animals were housed in a room maintained at 23 ± 1 °C with a 12 hr light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water were available *ad libitum.*

Genotyping

The genotype of offspring from β-endorphin knockout (-/-) mice (The Jackson Laboratory, Bar Harbor, ME, USA) was determined by polymerase chain reaction (PCR) using ear DNA obtained from pentobarbital (70 mg/kg, i.p.)-anesthetized mice. PCR analysis was performed using a BD Advantage™-GC 2 PCR kit (BD Biosciences Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions, with the extracted DNA template (10 ng/μL) and synthesized POMC primers: a sense primer of POMC (5'- GAA GTA CGT CAT GGG TCA CT -3'), an antisense primer of POMC (5'- GCT GGG GCA AGG AGG TTG AGA -3') and an antisense primer of phosphoglycerate kinase (5'-GGGAACTTCCTGACTAG GGG -3') [111], which includes a neo expression cassette inserted into a deleted exon 3 of the POMC gene [110]. PCR products generated using the primers of antisense phospho-glycerate kinase and sense

POMC revealed a single band of 850–900 base pairs (bp) (targeted deficient allele), and those generated using the primers of antisense POMC and sense POMC revealed a single band of 750–800 bp (wild-type allele). Briefly, a point mutation was introduced by site-directed mutagenesis into exon 3 of the POMC gene to generate a premature translational stop codon. The resultant truncated prohormone lacks the carboxyl-terminal 31 amino acids in β-endorphin, but is expressed at normal levels and is correctly processed into adrenocorticotropic hormone, melanocyte-stimulating hormones and gamma-lipotropic hormone.

Intrathecal (i.t.) injection

Intrathecal (i.t.) injection was performed following the method described in Chapter 1.

Measurement of thermal hyperalgesia

Thermal hyperalgesia was measured following the method described in Chapter 1.

Place conditioning

Place conditioning was conducted as described previously [112]. The apparatus was a shuttle box (15 cm wide × 30 cm long × 15 cm high) that was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor, and the other was black with a smooth floor to create equally preferable compartments. The place conditioning schedule consisted of three

phases (preconditioning test, conditioning, and postconditioning test). The preconditioning test was performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, a neutral platform was inserted along the seam separating the compartments, and animals that had not been treated with either drugs or saline were then placed on the platform. The time spent in each compartment during a 900 sec session was then recorded automatically with an infrared beam sensor (KN-80; Natsume Seisakusyo, Tokyo, Japan). Conditioning sessions (3 days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after s.c. injection of morphine (5mg/kg), these animals were placed in the opposite compartment to that in which they had spent the most time in the pre-conditioning test for 1 hr. On alternative days, these animals received saline and were placed in the other compartment for 1 hr. On the day after the final conditioning session, a postconditioning test that was identical to the preconditioning test was performed.

Pain-like behaviors

Groups of mice were placed individually in an observation cage (10×17×11 cm; width×length×height) immediately after i.t. injection of PDBu (10 nmol/mouse) or vehicle (10% DMSO in saline). The number of scratching and licking or biting behaviors was counted, and the duration of scratching and licking or biting behaviors was measured for 20 min after i.t. injection. In addition, the expression of severe tail-shaking and spontaneous or touch-evoked vocalization was checked for 20 min after i.t. injection. Each group consisted of 10 mice per vehicle or PDBu (10 nmol/ mouse)

treatment. All behavioral experiments were conducted in a single-blind fashion to avoid the effect of subjectivity.

Drugs

The drugs used in the present study were the specific PKC activator PDBu (Sigma Co., St Louis, Mo., USA) and morphine (Daiichi - Sankyo Co., Tokyo, Japan). PDBu was dissolved in 10% DMSO containing 0.9% saline, respectively. Morphine was dissolved in 0.9% saline.

Statistical data analysis

The data are presented as the mean ± S.E.M. The statistical significance of differences between the groups was assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni/Dunnett test or by Student's *t*-test. Fisher's exact probability test was used to compare two groups for a positive response.

Results

The genotype of offspring from β-endorphin knockout (-/–) mice was confirmed by PCR analysis using DNA extracted from the ear. As shown in Fig. 3-1A (lanes 1 and 2), β-endorphin knockout (–/–) mice yielded a targeted deficient allele single amplification product. In contrast, β-endorphin (+/+) (wild-type) mice showed a single amplification product (Fig. 3-1A, lanes 3 and 4).

Using these genotype mice, I investigated whether the PKC activator PDBu could affect the latency of paw withdrawal from a thermal stimulus at 24 hr after i.t. injection of PDBu. No differences in the response thresholds were noted before i.t. injection of vehicle or PDBu (Fig. 3-1B). The latency of paw withdrawal from a thermal stimulus was significantly decreased in PDBu-treated wild-type and β-endorphin knockout (–/–) mice (wild-PDBu-treated mice: 6.2 ± 0.2 sec, ***p<0.001 vs. wild-vehicle-treated mice; β-endorphin knockout (–/–)-PDBu-treated mice: 5.7 ± 0.3 sec, ###p<0.001 vs. β-endorphin knockout (–/–)-vehicle-treated mice, Fig. 3-1C) compared with that in vehicle-treated mice (wild-vehicle-treated mice: 9.7 ± 0.3 sec; β-endorphin knockout (–/–)-vehicle-treated mice: 9.6 ± 0.2 sec). Furthermore, a significant decrease in the latency of paw withdrawal from a thermal stimulus lasted for at least 9 days after i.t injection of PDBu (on day 9, wild-PDBu-treated mice: 6.3 ± 0.2 sec, ***p <0.001 vs. wild-vehicle-treated mice: 10.0 ± 0.3 sec, β-endorphin knockout-PDBu-treated mice: 6.1 ± 0.2 sec, ###p <0.001 vs. β-endorphin knockout-vehicle-treated mice; 9.5 ± 0.2 sec, Fig. 3-1D). However, these were no

significant changes in the response thresholds between wild-type and β-endorphin knockout mice.

In the present study, I also observed pain-like behaviors induced by i.t. injection of PDBu in wild-type or β-endorphin knockout mice. PDBu (10 nmol/mouse) given i.t. produced dramatic scratching and licking or biting behaviors in mice with both genotypes (Table 3-1). These PDBu-induced scratching and licking and biting behaviors started about 4–5 min after i.t. injection and lasted for approximately 60 min. Control mice that were injected i.t. with vehicle (10% DMSO in saline) did not show these behaviors (Table 3-1). Furthermore, other aversive behaviors such as severe tail-shaking and vocalization were also observed after i.t. administration of PDBu (Table 3-1). However, there were no significant differences in pain-like behaviors between wild-type and β-endorphin knockout mice.

Recently, it has been reported that activated PKC in the spinal cord under chronic pain-like hyperalgesia may play a substantial role in the suppression of the morphine-induced rewarding effect in mice with chronic pain-like hyperalgesia [113]. Therefore, I next investigated whether β-endorphin could be involved in the suppression of the morphine-induced rewarding effect by i.t. pretreatment with PDBu using β-endorphin knockout (–/–) mice. The s.c. injection of morphine (5 mg/kg) produced a significant place preference in mice of both genotypes that had been pretreated i.t. with vehicle (***$p<0.001$ vs. wild-type or **$p<0.01$ vs. β-endorphin knockout with i.t. vehicle plus s.c. saline group). A single i.t. pretreatment with PDBu (10 nmol/mouse) in wild-type mice caused a significant suppression of the morphine-induced place

preference (##p<0.01; wild-type with i.t. vehicle plus s.c. morphine group vs. wild-type with i.t. PDBu (10 nmol/mouse) plus s.c. morphine group, Fig. 3-2). In contrast, the place preference induced by morphine at 5 mg/kg (s.c.) was not suppressed in β-endorphin knockout (–/–) mice with a single i.t. pretreatment of PDBu at 10 nmol (Fig. 3-2).

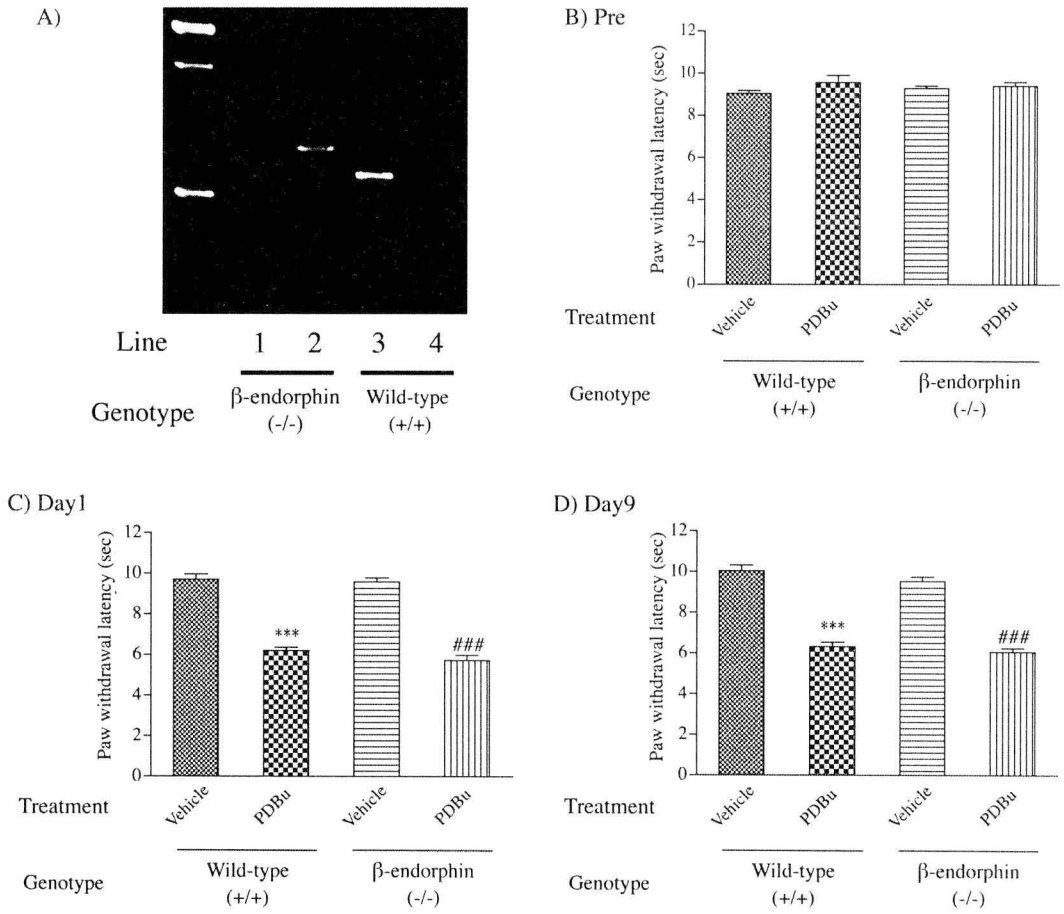


Fig. 3-1

Effect of a single (i.t.) injection of the PKC activator PDBu on the latency of paw withdrawal from a thermal stimulus in wild-type (+/+) and β-endorphin knockout (–/–) mice. (A) Representative PCR for using *propiomelanocortin (Pomc)* DNA extracted from the ear of either wild-type (+/+) or β-endorphin knockout (–/–) mice. While β-endorphin knockout (–/–) yielded a targeted deficient allele single amplification product (lanes 1 and 2), wild-type mice showed a wild-type allele single amplification products (lanes 3 and 4). The latency of paw withdrawal from a thermal stimulus was measured at (B) 0, (C) 1, and (D) 9 days after i.t. injection of vehicle or PDBu (10 nmol/mouse). Each column represents the mean with SEM of 9–10 mice. ***p<0.001 vs. wild-type i.t. vehicle group, ###p<0.001 vs. β-endorphin knockout i.t. vehicle group.

Pain-like behaviors induced by i.t. injection of PDBu in β-endorphin (-/-) mice

Aversive behavior	Wild-type (+/+)		β-endorphin (-/-)	
	Vehicle	PDBu	Vehicle	PDBu
Scratching(>10 times)	0/10	5/10 **	0/10	6/10 ##
Scratching(>1 min)	0/10	4/10 *	0/10	3/10
Licking or biting (>10 times)	1/10	8/10 ***	2/10	8/10 ###
Licking or biting (>1 min)	0/10	9/10 ***	0/10	8/10 ###
Severe tail-shaking	0/10	10/10 ***	0/10	7/10 ##
Vocalization	0/10	6/10 **	0/10	4/10 #

Table 3-1

Groups were wild-type mice treated i.t. with PDBu (10 nmol/mouse) or vehicle (10% DMSO in saline) and β-endorphin knockout (–/–) mice treated i.t. with PDBu (10 nmol/mouse) or vehicle (10% DMSO in saline). Immediately after i.t. injection, the number of scratching and licking or biting behaviors was counted and the duration of scratching and licking or biting behaviors was measured for 20 min after i.t. injection of PDBu. In addition, the expression of severe tail shaking and spontaneous or touch-evoked vocalization was checked for 20 min after the i.t. injection of PDBu. The term 'positive animal' refers to animals that exhibited a certain number of positive responses (>10 times) or showed a threshold total duration of responses (>1 min). *p<0.05, **p<0.01 and ***p<0.001 vs. wild-type i.t. vehicle group. #p<0.05, ##p<0.01 and ###p<0.001 vs. β-endorphin knockout i.t. vehicle group.

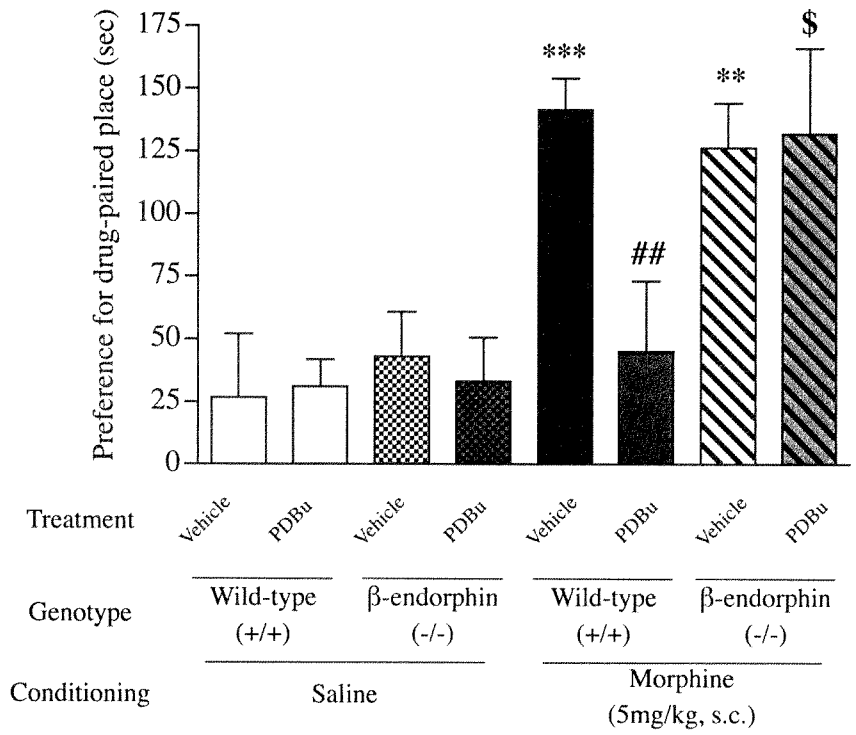


Fig. 3-2

Effects of a single i.t. injection of PDBu on the morphine-induced place preference in wild-type (+/+) and β-endorphin knockout (–/–) mice. Conditioning sessions (three for morphine, three for saline) were started the day after i.t. injection of PDBu (10 nmol/mouse) or vehicle and conducted once daily for 6 days. Immediately after each s.c. injection of morphine (5 mg/kg) or saline, the mouse was placed and conditioned in either compartment for 1 hr. On the day after the final conditioning session, a post-conditioning test that was identical to the pre-conditioning test was performed. The ordinate shows mean differences between times spent in the morphine-paired and saline-paired sides of the test box. The data represent the mean with SEM of 7–10 mice. **p<0.01 and ***p<0.001 vs. wild-type i.t. vehicle plus s.c. saline group. ##p<0.01 vs. wild-type i.t. vehicle plus s.c. morphine group. $p<0.05 vs. wild-type i.t. PDBu plus s.c. morphine group.

Discussion

Recent studies have provided evidence that PKC expressed on dorsal horn neurons plays an important role in regulating pain hypersensitivity in several different pain models [31,89,114,115]. The activation of PKC in the dorsal horn of the spinal cord may be responsible for the release of excitatory amino acids and neuropeptides, resulting in the initiation of central sensitization. It was documented that thermal hyperalgesia induced by sciatic nerve ligation was markedly suppressed by repeated i.t. pretreatment with a selective PKC inhibitor [116]. It was also found that the level of membrane-bound PKCγ isoform, which is identified in neurons of the brain and the inner part of laminae II of the spinal cord, was significantly increased on the ipsilateral side of the spinal cord in sciatic nerve-ligated mice [107]. Interestingly, mice that lack the PKCγ isoform exhibit normal responses to acute pain stimuli, but also show an almost complete lack of development of neuropathic pain-like behaviors after sciatic nerve ligation [30,31]. Collectively, these findings provide further evidence that the activation of neuronal cPKC in the dorsal horn of the spinal cord by nerve injury may play a key role in the development of a neuropathic pain-like state in mice.

The key finding of the present study was that suppression of the morphine-induced rewarding effect by i.t. injection of PDBu was abolished in β-endorphin knockout mice. Previously it has been demonstrated that a single i.t. injection of PDBu caused a dramatic suppression of the morphine-induced place preference [28]. Nociceptive information is thought to be transmitted by neuronal pathways projecting from the

thalamus to various brain regions, including the VTA, which possesses high densities of μ-opioid receptors and plays a critical role in psychological dependence on μ-opioid receptor agonists such as morphine. Interestingly, it has been reported that sciatic nerve injury leads to a reduction in μ-opioidergic function in the VTA, resulting in the suppression of morphine-induced dopamine release in the N.Acc., which is terminus of the mesolimbic dopaminergic neurons [65].

Based on these findings, the direct activation of PKC in the spinal cord could lead to an increase in the release of β-endorphin in the brain. This phenomenon may eventually cause a reduction in μ-opioid receptor function in the VTA, resulting in suppression of the morphine-induced rewarding effect under a neuropathic pain-like state.

In the present study, there were no differences in the response to thermal stimuli or pain-like behavior induced by i.t. PDBu between wild-type and β-endorphin knockout mice. In contrast, the direct activation of PKC by i.t. PDBu in the spinal cord eliminated the suppression of the morphine-induced place preference following sciatic nerve ligation in β-endorphin knockout mice. Although further study is required, it is possible that the release of β-endorphin by the activation of PKC in the spinal cord can regulate the affective-motivational component of pain, which includes mesolimbic dopaminergic transmission, but not the sensory-discriminative component of pain. This hypothesis can be supported by the recent finding that sciatic nerve ligation causes a reduction in μ-opioid receptor function with an increase in the level of membrane-bound G-protein-coupled kinase 2, which can mediate the desensitization of μ-opioid receptors. This phenomenon was observed in the VTA but not in the

thalamus, PAG or pons/medulla [66,117].

In conclusion, I have demonstrated that deletion of the β-endorphin gene reversed the suppression of the morphine-induced place preference by the activation of spinal PKC by i.t. PDBu treatment in mice. These findings provide further evidence that a neuropathic pain-like state caused by the direct activation of PKC in the spinal cord leads to the constant release of β-endorphin at the supraspinal level, resulting in the suppression of psychological dependence on opioids.

Chapter 4

Direct evidence for the involvement of endogenous β-endorphin in the suppression of the morphine-induced rewarding effect under a neuropathic pain-like state

Introduction

The mesolimbic dopamine system, which consists of dopaminergic neurons in the VTA and their projections to the N.Acc, has been implicated in the rewarding properties of opioids [108]. Especially, μ-opioid receptors located in the VTA have been shown to be critical for opioid reward [118,119]. Injection of the specific μ-opioid receptor agonist DAMGO into the VTA, but not the N.Acc., produced a significant place preference in the rat [108].

When a patient is an appropriate candidate for an opioid but the drug is not available in a sufficient dose to allow the patient to function adequately and maintain a reasonable lifestyle, it has often been proposed that opioid addiction does not arise as a consequence of the treatment of pain with opioid. In fact, this argument has been used to support the safe use of opioid for the treatment of severe pain [61,62]. Although many clinical studies have demonstrated that when opioid analgesics including morphine are used to control pain, psychological dependence is not a major concern [120], the scientific support for this clinical experience is still needed if physicians are to be confident in using adequate doses of opioids for the treatment of severe pain.

Previously, it has been reported that morphine failed to induce rewarding effects in rodents under formalin injection [63,67,69] and sciatic nerve ligation [65,66,107]. In addition, sciatic nerve ligation suppressed the morphine-induced dopamine release in the N.Acc. associated with the inhibition of μ-opioid receptor-mediated G-protein activation in the

VTA [66]. These findings suggest that the dysfunction of μ-opioid receptor in the VTA may be responsible for the suppression of morphine dependence under a chronic pain.

β-Endorphin is an endogenous opioid peptide that has a profound analgesic effect when administered to the central nervous system [121,122]. The acute release of β-endorphin into ventricular CSF has been demonstrated during electrical stimulation of the periaqueductal grey matter in humans, and a relation has been found between analgesia and β-endorphin release [123]. While most studies on patients with chronic pain have found no differences in CSF β-endorphin concentrations, a few have shown decreased β-endorphin concentrations during chronic pain. Therefore, there is no firm evidence obtained from measuring β-endorphin in human CSF that the presence of chronic pain in general is associated with altered activity in β-endorphin neurons. In rodents, β-endorphin levels have been found to be increased in the rat hypothalamus, periaqueductal grey matter and posteromedial thalamus under formalin-induced pain [124-126]. Furthermore, it has been reported that pain induces the release of endogenous opioid peptides in the brain, including the mesolimbic area in humans [127]. Therefore, I proposed that sciatic nerve injury may increase the release of endogenous opioid peptides that interact with μ-opioid receptors in the mesolimbic area, and in turn cause the down-regulation of μ-opioid receptor in this region.

Many lines of evidence support the notion that the activation of spinal PKC is closely related to the development or maintenance of neuropathic pain. In Chapter 3, I found that released β-endorphin is likely to be responsible for the development of the

suppression of morphine-induced rewarding effect following a direct activation of spinal PKCγ.

Knockout mice with β-endorphin gene deletions have been successfully developed by homologous recombination [128]. Transgenic β-endorphin knockout mice allow us to determine physiological functions mediated via central β-endorphin-containing fibers under a chronic pain-like state. The aim of the present study was to further investigate the molecular mechanisms that underlie the suppression of the opioid-induced rewarding effect under a neuropathic pain-like state using β-endorphin knockout mice.

Materials and Methods

Animals

As described in Chapter 3, I used the male and female β-endorphin peptides derived from *proopiomelanocortin (Pomc)* gene-knockout mice (The Jackson Laboratory, Bar Harbor, ME, USA) and male Sprague-Dawley rats (Tokyo Laboratory Animals Science Co., Ltd, Tokyo, Japan). Animals were housed in a room maintained at 23 ± 1 °C with a 12 hr light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water were available *ad libitum.*

Genotyping

The procedure for genotyping was performed following the method described in Chapter 3.

Surgery and microinjection

After 3 days of habituation to the main animal colony, all rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The anesthetized animals were placed in a stereotaxic apparatus. The skull was exposed, and a small hole was made using a dental drill. A guide cannula (AG-9; Eicom, Kyoto, Japan) was implanted into the VTA (from bregma: posterior, 5.3 mm; lateral, 0.9 mm; ventral, 7.7 mm) according to the atlas of Paxinos and Watson (1998) [129]. The guide cannula was fixed to the skull with cranioplastic cement. Three to five days after surgery, the animals were injected

with β-endorphin antibody (1:500 or 1:1000) (Peninsula Laboratories Inc., CA) diluted in saline or saline alone in a volume of 0.3 μL/rat into the VTA 10 min before the start of nerve injury and once a day for 3 consecutive days after nerve injury. In the microinjection method, I used an injection cannula (AMI-9.5; Eicom) that extended beyond the guide cannula by 0.5 mm. A stainless steel injection cannula was inserted into the guide cannula for each animal. The injection cannula was connected through polyethylene tubing to a 10 μL Hamilton syringe that was preloaded with β-endorphin antibody or saline, which was delivered by a motorized syringe pump in a volume of 0.3 μL over 60 sec.

Neuropathic pain model

The rats and mice were deeply anesthetized with isoflurane. A partial sciatic nerve injury by tying a tight ligature with a 8–0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30, Olympus, Tokyo, Japan) as described previously [130,131]. In sham-operated animals, the nerve was exposed without ligation.

Place conditioning

Place conditioning was conducted as described previously [112]. The apparatus was a shuttle box (mouse size, 15 cm wide × 30 cm long × 15 cm high; rat size, 30 cm wide × 60 cm long × 30 cm high) that was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor,

and the other was black with a smooth floor to create equally preferable compartments. The place conditioning schedule consisted of three phases (preconditioning test, conditioning, and postconditioning test). The preconditioning test was performed as follows: the partition separating the two compartments was raised to 7 cm (mouse) or 12 cm (rat) above the floor, a neutral platform was inserted along the seam separating the compartments, and animals that had not been treated with either drugs or saline were then placed on the platform. The time spent in each compartment during a 900 sec session was then recorded automatically with an infrared beam sensor (KN-80; Natsume Seisakusyo, Tokyo, Japan). Conditioning sessions (3 days for DAMGO (Sigma Co., St Louis, MO, USA) (rats) or morphine (mice), 3 days for saline) were conducted once daily for 6 days. Immediately after the microinjection of DAMGO into the VTA (rats) or s.c. injection of morphine (5mg/kg) (mice), these animals were placed in the compartment opposite that in which they had spent the most time in the pre-conditioning test for 1 hr. On alternative days, these animals received saline and were placed in the other compartment for 1 hr. On the day after the final conditioning session, a postconditioning test that was identical to the preconditioning test was performed.

Measurement of thermal hyperalgesia

To assess the sensitivity to thermal stimulation, each of the hind paws of rats were tested individually using a thermal stimulus apparatus (model 33 Analgesia Meter; IITC Inc./ Life Science Instruments, Woodland Hills, CA, USA). The intensity of the

thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 8 to 10 sec in naive rats. Only quick hind paw movements (with or without licking of the hind paws) away from the stimulus were considered to be a withdrawal response. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured alternating between the left and right with an interval of more than 3 min between measurements. The latency of paw withdrawal after the thermal stimulus was determined as the average of three measurements per paw.

Injections of retrograde marker fluoro-gold

To identify mesolimbic projecting neurons, the retrograde tracer fluoro-gold (FG) (4%, Fluorochrome, Englewood, CO) was injected into the N.Acc. First, rats were deeply anesthetized with isoflurane. The anesthetized animals were placed in a stereotaxic apparatus. The skull was exposed, and double holes were drilled through the skull over the N.Acc. (AP: +1.5 mm, ML: -0.5 mm, DV: –7.0 mm) according to the atlas of Paxinos and Watson (1998) [129]. The skull was exposed, and a small hole was made using a dental drill. A micropipette with a diameter of about 15-20 μm was filled with FG solution. FG was pressure-injected (200 nL) into the N.Acc. of the right hemisphere. After the injection, the micropipette was left in place for 5 min to avoid leakage after removal of the injection cannula. Following surgery, the scalp was closed. Three days after FG injection, rats were re-anesthetized with isoflurane and subjected to surgery for partial nerve injury.

Sample preparation

Ten days after nerve ligation, rats were deeply anesthetized with isoflurane and intracardially perfusion-fixed with freshly prepared 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the brains were then quickly removed, and thick coronal sections of the midbrain including the VTA was initially dissected using Brain Blocker was quickly removed. These sections were post-fixed in 4 % paraformaldehyde for 3 hr, and permeated with 20 % sucrose in 0.1M PBS for 1 day and 30 % sucrose in 0.1M PBS for 2 days with agitation. They were then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at –30 ℃ until use. Frozen these sections were cut with a freezing cryostat (Leica CM 1510, Leica Microsystems AG, Wetzlar, Germany) at a thickness of 8 mm and thaw-mounted on poly-L-lysine-coated glass slides.

Immunohistochemistry

The brain sections were blocked in 10 % normal goat serum (NGS) in 0.01M PBS for 1 hr at room temperature. The primary antibody [1:400 phosphorylated-tyrosine hydroxylase (Ser31) (Chemicon International, Inc., CA, USA)] was diluted in 0.01 M PBS containing 10 % NGS and incubated for 2 nights at 4 °C. The samples were then rinsed and incubated with an appropriate secondary antibody conjugated with Alexa 488 for 2 hr at room temperature. Since the staining intensity might vary between

experiments, control sections were included in each run of staining. The slides were then cover-slipped with PermaFluor Aqueous mounting medium (Immunon™; ThermoShandon, Pittsburgh, PA, USA). Fluorescence of FG and immunolabeling were detected using a light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus). Fluorescence immunolabeling was detected using a U-MNIBA filter cube (Olympus, Tokyo, Japan). The distribution of gold-emitting FG retrogradely-labeled neurons and FG injection sites was determined using a microscope (Olympus BX-60; Olympus). The fluorescence of FG was observed with a filter (excitation/emission peaks, 323/408 nm).

[^{35}S]GTPγS binding assay

For membrane preparation, the mouse a section of the lower midbrain that included the VTA and limbic forebrain that included the N.Acc, as described previously [132], were quickly removed after decapitation, and rapidly transferred to a tube filled with ice-cold buffer. The membrane homogenate (3–8 μg protein/assay) was prepared as described previously [133] and incubated at 25℃ for 2 hr in 1mL of assay buffer with various concentrations of each agonist, 30 μM GDP and 50pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by filtration using Whatman GF/B glass filters (Brandel, Gaithersburg, MD, USA) that had been presoaked in 50 μM Tris-HCl, pH 7.4, and 5 μM $MgCl_2$ at 4℃ for 2 hr. The filters were washed three times with 5 mL of ice-cold Tris-HCl buffer, pH 7.4, and then transferred to scintillation-counting vials containing 3 mL of Clear-sol II (Nacalai

Tesque Inc., Kyoto, JAPAN) and equilibrated for 12 hr. The radioactivity in the samples was determined with a liquid scintillation analyzer. Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. In the present study, sample preparation was performed 10 days after partial sciatic nerve-ligation.

Mouse *in vivo* microdialysis study and quantification of dopamine

Stereotaxic surgery was performed under sodium pentobarbital (70 mg/kg, i.p.) anesthesia. Mice were placed in a stereotaxic apparatus, and the skull was exposed. A small hole was then made using a dental drill. A microdialysis probe (D-I-6-01; 1mmmembrane length; Eicom) was implanted into the N.Acc. (from bregma: anterior, +1.5 mm; lateral, +0.9 mm; ventral,-4.9 mm) according to the atlas of Paxinos and Franklin (1997) [105]. The microdialysis probe was fixed to the skull with cranioplastic cement. At 24 hr after implantation, mice were placed in the experimental cages (30 cm wide × 30 cm long × 30 cm high). The probe was perfused continuously at a flow rate of 2 μL/min with aCSF containing 0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl, and 1.2 mM $CaCl_2$. Outflow fractions were taken every 5 min. After 15 baseline fractions were collected, mice were given morphine (10 mg/kg, s.c.) or saline (1 mL/kg, s.c.). For this experiment, dialysis samples were collected for 180 min after morphine or saline treatment. Dialysis fractions were then analyzed using HPLC with ECD (HTEC-500; Eicom). Dopamine was separated by a column with a mobile phase containing 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 2.0 mM sodium 1-decane sulfonate, 0.1mM EDTA (2Na), and 1% methanol. The mobile phase was delivered at a flow

rate of 550 μL/min. Dopamine was identified according to the retention times of a dopamine standard, and amounts of dopamine were quantified by calculating with peak areas. The baseline microdialysis data were calculated as concentrations in the dialysates. Other microdialysis data are expressed as percentages of the corresponding baseline level.

Drugs

The drug used in the present study were morphine (Daiichi-Sankyo Co., Tokyo, Japan) and DAMGO (Sigma Co., St Louis, Mo., USA). Morphine and DAMGO were dissolved in 0.9% saline.

Statistical data analysis

The data are presented as the mean ± S.E.M. The statistical significance of differences between the groups was assessed by one- or two-way ANOVA followed by the Bonferroni/Dunnett test or by Student's *t*-test.

Results

Thermal hyperalgesia induced by sciatic nerve ligation in rats

First, I confirmed that sciatic nerve-ligated rats exhibited a significant and persistent decrease in the latency of paw-withdrawal on the ipsilateral side, indicating a neuropathic pain-like state (Fig. 4-1). Since the withdrawal latencies of the ipsilateral paw in response to a thermal stimulus were maximally decreased at 4 days after sciatic nerve ligation (Day 4: sham-ipsilateral vs. ligation-ipsilateral, $p<0.001$), these animals were used for behavioral and biochemical studies.

Effect of intra-VTA injection of β-endorphin antibody on suppression of the DAMGO-induced place preference by sciatic nerve ligation in rats

Under these conditions, I investigated whether sciatic nerve ligation could affect the place conditioning induced by the selective μ-opioid receptor agonist DAMGO in rats. It has been reported that intra-VTA administration of DAMGO caused a dose-dependent preference for the drug-associated place in mice [108]. In the present study, I also demonstrated that the microinjection of DAMGO at 1 nmol into the VTA produced a significant preference for the drug-associated place in sham-operated rats ($p<0.05$ vs. sham-saline groups) (Fig. 4-2). In contrast, the DAMGO-induced place preference was significantly suppressed in sciatic nerve-ligated rats ($p<0.05$ vs. sham-DAMGO groups). In contrast, microinjection of β-endorphin antibody into the VTA just before and after 3 days of sciatic nerve-ligation significantly reversed the

suppression of DAMGO-induced place preference in rats with sciatic nerve ligation ($p<0.01$ vs. saline-ligation-DAMGO groups).

Effect of sciatic nerve ligation on the activity of the mesolimbic dopamine system in rats

To investigate a possible change in tyrosine hydroxylase activity in the VTA of sciatic nerve-ligated rats, immunohistochemical studies were performed. As shown in Fig. 18, p-TH (Ser31) immunoreactivity was prominently observed in the VTA of sham-operated rats (Fig. 4-3A and D: high magnification). Sciatic nerve ligation dramatically diminished the p-TH immunoreactivity in this region (Fig. 4-3B). Consistent with many previous studies which have characterized afferent projections to the N.Acc., FG-containing cells were apparently detected in the VTA after microinjection of the retrograde tracer FG into the shell region of the unilateral N.Acc. (Fig. 4-3C). Further immunostaining showed that a population of retrogradely labeled neurons in the VTA was also immunoreactive for p-TH (Ser31) (Fig.4-3E).

Effect of deletion of the β-endorphin gene on the suppression of morphine reward by sciatic nerve ligation in mice

The genotype of offspring from β-endorphin knockout (-/-) mice was confirmed by PCR analysis using DNA extracted from the ear. As shown in Fig. 4-4A (lanes 3 and 4), β-endorphin knockout (-/-) mice yielded a targeted deficient allele single amplification product. In contrast, β-endorphin (+/+) (wild-type) mice showed a single amplification

product (Fig. 4-4A, lanes 1 and 2). Using these genotype mice, I performed a conditioned place preference test. In wild-type mice, the morphine-conditioned place preference was significantly attenuated following sciatic nerve ligation. ($p<0.05$ vs. sham-wild-type-morphine; Fig. 4-4B). In contrast, there were no significant changes in the morphine-induced place preference between sham- and nerve-ligated β-endorphin knockout (-/-) mice (Fig. 4-4B), indicating that suppression of the morphine-induced rewarding effect in sciatic nerve ligation was abolished by absence of the β-endorphin gene.

Effect of the deletion of β-endorphin gene on the down-regulation of μ-opioid receptor function in the VTA of mice with sciatic nerve ligation

The ability of DAMGO to activate μ-opioid receptor linked to its G-proteins in the lower midbrain including the VTA obtained from sham-operated or sciatic nerve-ligated wild-type and β-endorphin knockout (-/-) mice was examined by monitoring the binding of [^{35}S]GTPγS. DAMGO (10^{-8}–10^{-5} M) produced a concentration-dependent increase in the binding of [^{35}S]GTPγS to lower midbrain membranes in sham-operated mice. Conversely, the level of [^{35}S]GTPγS binding to this area stimulated by DAMGO in nerve-ligated wild-type mice was significantly lower than that observed in sham-operated wild-type mice (Fig. 4-5A, 10^{-5} M; $p<0.01$ vs. sham-operated wild-type group). In contrast, there were no significant changes in the DAMGO-induced increase in the binding of [^{35}S]GTPγS to lower midbrain membranes between nerve-ligated

β-endorphin knockout (-/-) mice and sham-operated β-endorphin knockout (-/-) mice (Fig.4-5B).

Since it has been demonstrated that activation of the mesolimbic dopamine system is critically linked to the expression of the rewarding effects of morphine, I examined whether the suppression of the rewarding effect induced by morphine after nerve injury could result from changes in dopamine receptor function linked to G-proteins in the N.Acc. (Fig. 4-6). The ability of dopamine to activate G-proteins in the N.Acc. of sham-operated and sciatic nerve-ligated mice was examined by monitoring the binding of [^{35}S]GTPγS to N.Acc. membranes. dopamine (10^{-8}–10^{-5} M) produced a concentration-dependent increase in [^{35}S]GTPγS binding to N.Acc. membranes from both sham-operated and sciatic nerve-ligated wild-type mice to the same degree (Fig. 4-6A). Similarly, no differences in the increase in [^{35}S]GTPγS binding stimulated by dopamine were noted between sham-operated and sciatic nerve-ligated β-endorphin knockout (-/-) mice (Fig. 4-6B).

Changes in the increase in the dialysate dopamine level induced by morphine in sciatic nerve-ligated β-endorphin knockout mice

Fig. 4-7*A* shows the location of microdialysis probes within the mouse N.Acc. Probe-inserted regions were localized in the N.Acc. Only data from mice in which probes had been accurately inserted in the N.Acc. were used for subsequent statistical analysis. The effect of the s.c. administration of morphine on the dialysate dopamine level in the mouse N.Acc. is shown in Fig. 4-7B. The dopamine levels were markedly

increased by s.c. injection of morphine at 10 mg/kg compared with that induced by saline treatment in sham-operated wild-type mice. Under these conditions, the increased level of dialysate dopamine in the N.Acc. stimulated by morphine was significantly decreased in sciatic nerve-ligated wild-type mice ($F(1, 234) = 2.824$; $p<0.0001$, sham-operated wild-type mice treated with morphine vs. ligated wild-type mice treated with morphine). However, the increase in the level of dialysate dopamine stimulated by morphine was not affected in sciatic nerve-ligated β-endorphin knockout (-/-) mice.

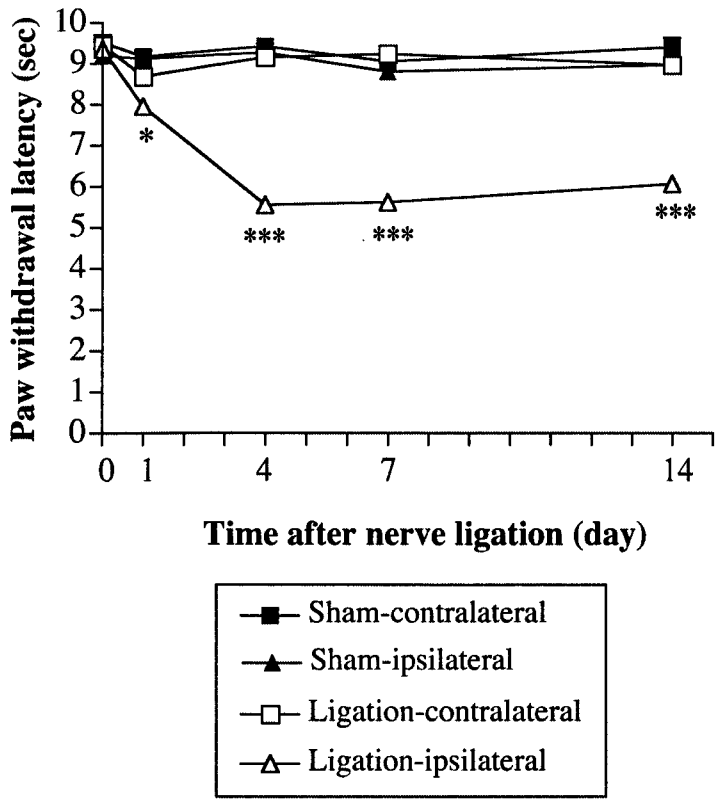


Fig. 4-1

Effect of sciatic nerve ligation on withdrawal responses to thermal stimulation. There was no difference in the basal response between sham-operated and sciatic nerve-ligated rats before surgery (day 0). Thermal hyperalgesia was only observed on the ipsilateral side of sciatic nerve-ligated rats. The data are presented as the mean ± S.E.M. of 6 rats. *$p<0.05$ and ***$p<0.001$ vs. Sham group.

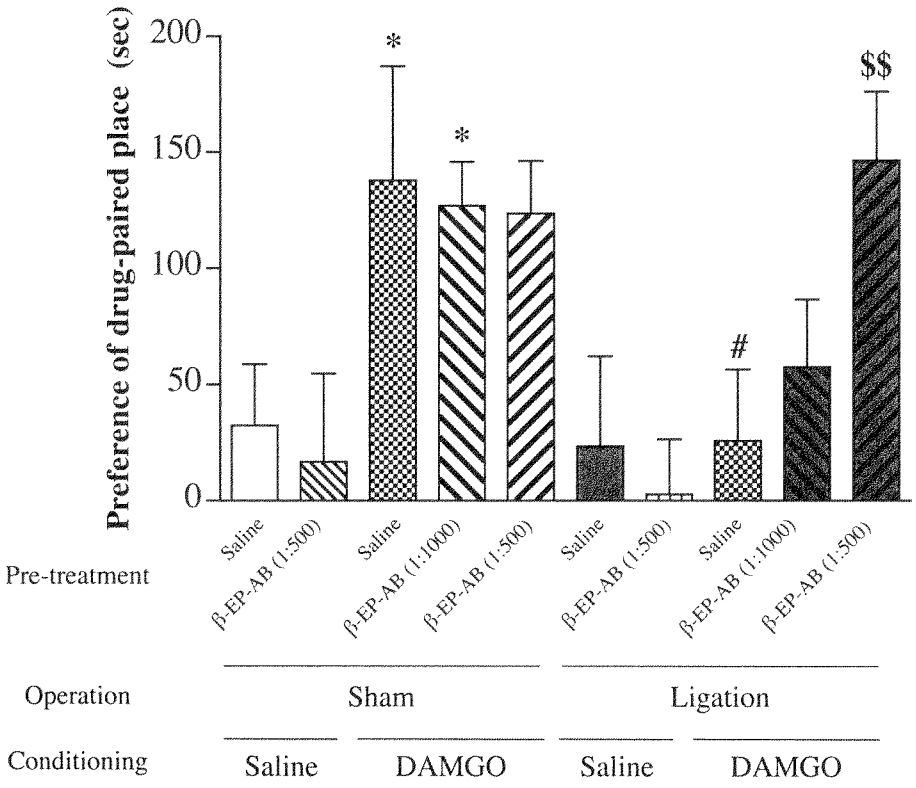


Fig. 4-2

Effect of microinjection of a specific antibody to β-endorphin into the VTA on the suppression of a place preference induced by DAMGO under a neuropathic pain-like state. Ordinate: mean differences (sec) between time spent in the DAMGO- and saline-paired sides of the test box. Each column represents the mean conditioning score with S.E.M. of 6-7 rats. *$p<0.05$ vs. Saline/sham/saline group. #$p<0.05$ vs. Saline/sham/DAMGO group. \$\$$p<0.01$ vs. Saline/ligation/DAMGO group.

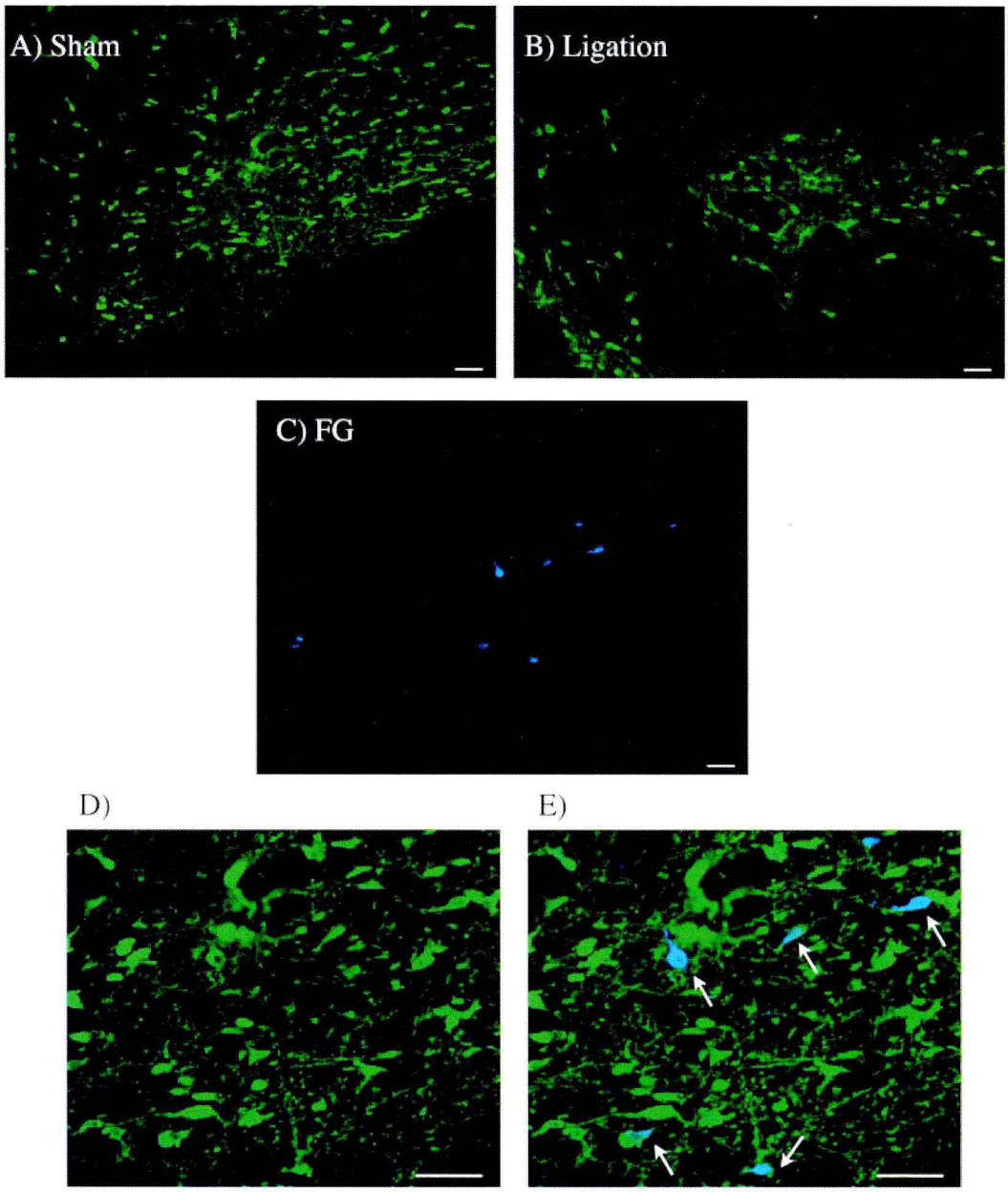


Fig. 4-3

Immunofluorescent staining for phosphorylated-tyrosine hydroxylase (Ser31) (p-TH)-like immunoreactivity (IR) in the VTA of sham-operated rats or nerve-ligated rats (A). The images show p-TH-like IR in the VTA of nerve-ligated rats (B) or sham-operated rats (A). (C-E) Projection from the VTA to the nucleus accumbens (N.Acc.). (C) Cells in the VTA after microinjection of FG into the N.Acc. are shown. The cell is positive for FG. (D and E) Apparent co-localization of p-TH (green) with fluoro-gold (blue) in the VTA. The sample was prepared 10 days after nerve ligation in rats. Scale bars = 50 μm

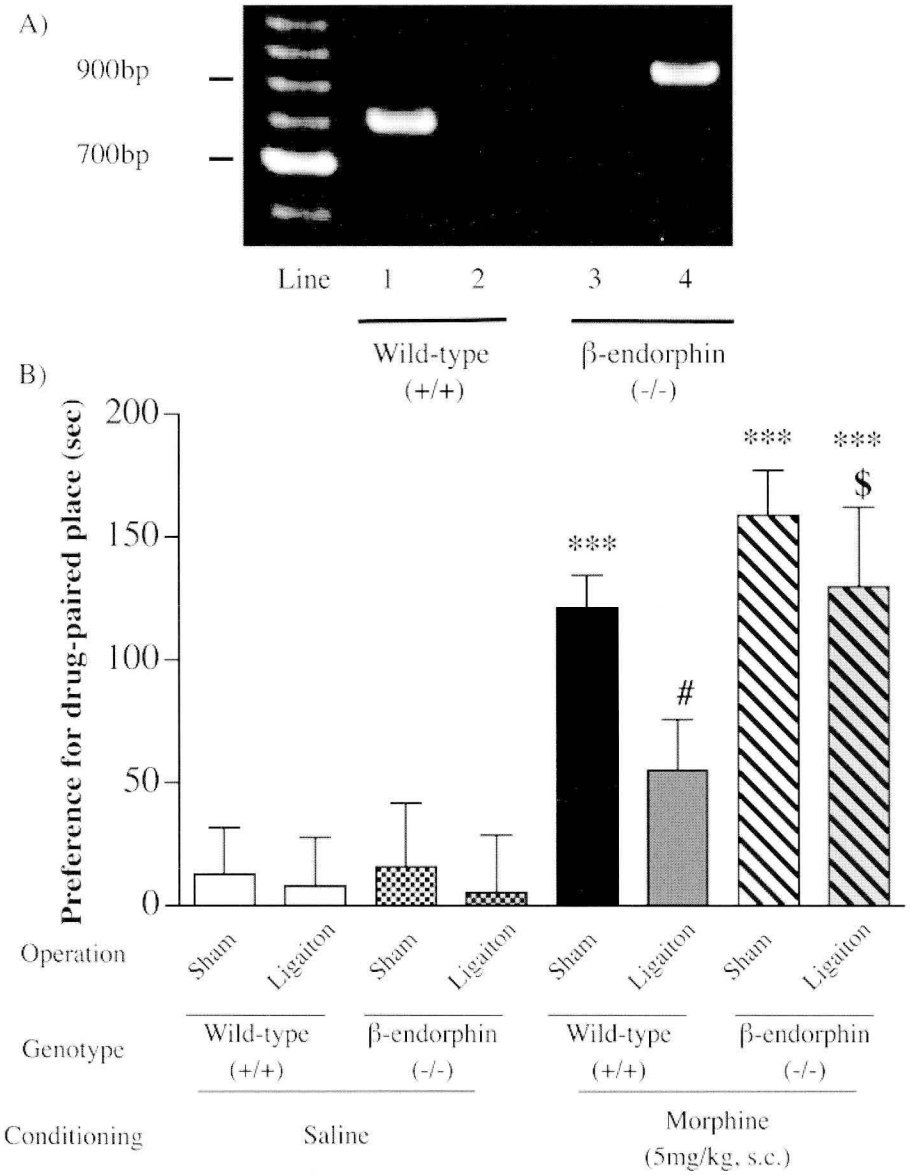


Fig. 4-4

(A) Representative PCR for *proopiomelanocortin (Pomc)* DNA extracted from the ear of either wild-type (+/+) or β-endorphin knockout (–/–) mice. β-Endorphin knockout (–/–) yielded a targeted deficient allele single amplification product (lanes 3 and 4), whereas wild-type mice showed a wild-type allele single amplification products (lanes 1 and 2). (B) Disappearance of the suppression of the morphine-induced place preference by sciatic-nerve ligation due to the absence of the β-endorphin gene. Both genotypes of mice were conditioned by saline or morphine (5 mg/kg, s.c.) after sciatic-nerve ligation or sham-operation. The data represent the mean with S.E.M. of seven to eight mice. ***$p<0.001$ vs. Sham/wild-type/saline group. #$p<0.05$ vs. Sham/wild-type/morphine group. \$$p<0.05$ vs. Ligation/wild-type/morphine group.

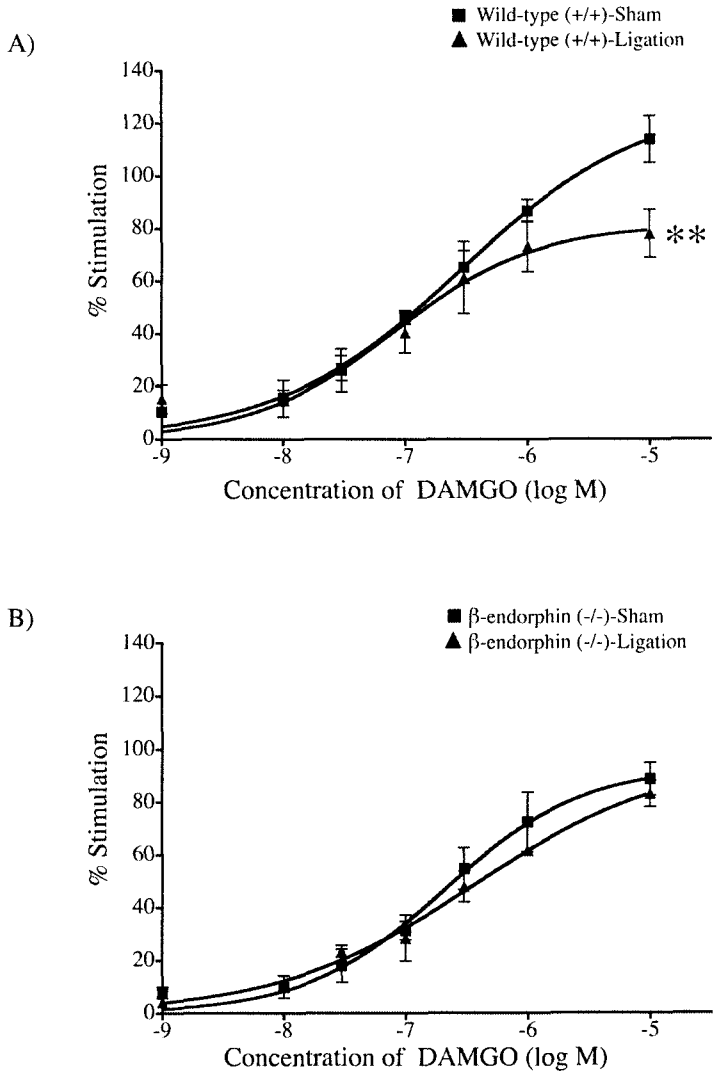


Figure 4-5

Concentration-response curve of DAMGO on the binding of [^{35}S]GTPγS to membranes of the lower midbrain from (A) wild-type (+/+) and (B) β-endorphin knockout (–/–) mice after sciatic nerve-ligation or sham-operation. Each value represents the mean ± S.E.M. of 6 samples. **p< 0.01 vs. Sham-wild-type group.

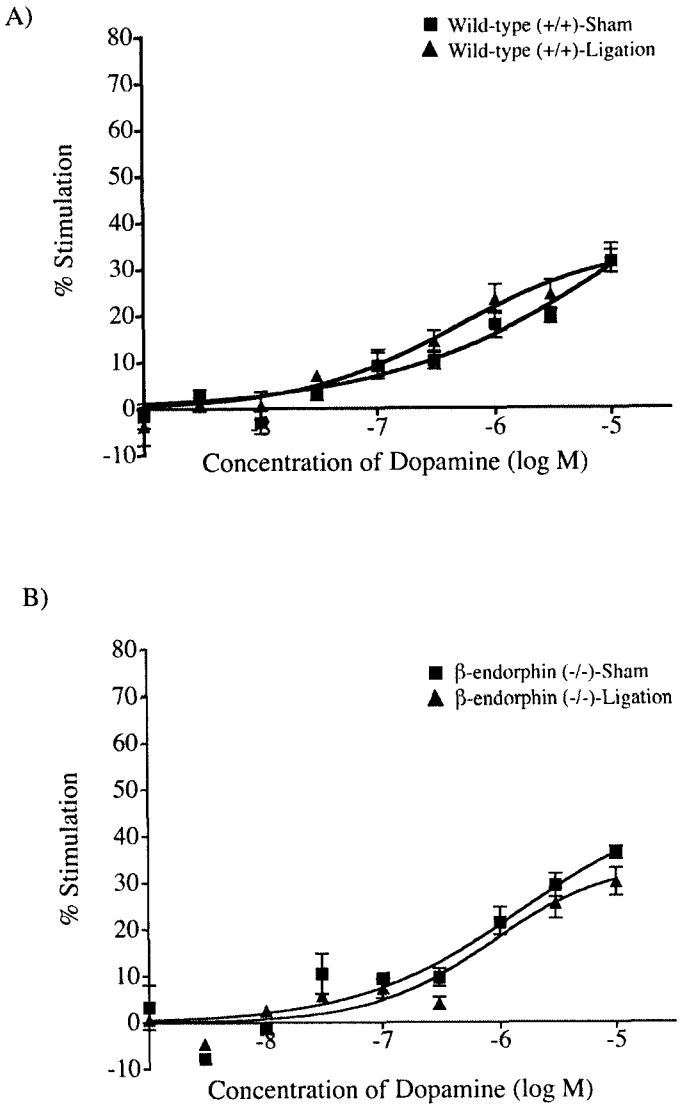


Figure 4-6

Concentration-response curve of dopamine on the binding of [^{35}S]GTPγS to membranes of the lower midbrain from (A) wild-type (+/+) and (B) β-endorphin knockout (–/–) mice after sciatic nerve-ligation or sham-operation. Each value represents the mean ± S.E.M. of 6 samples.

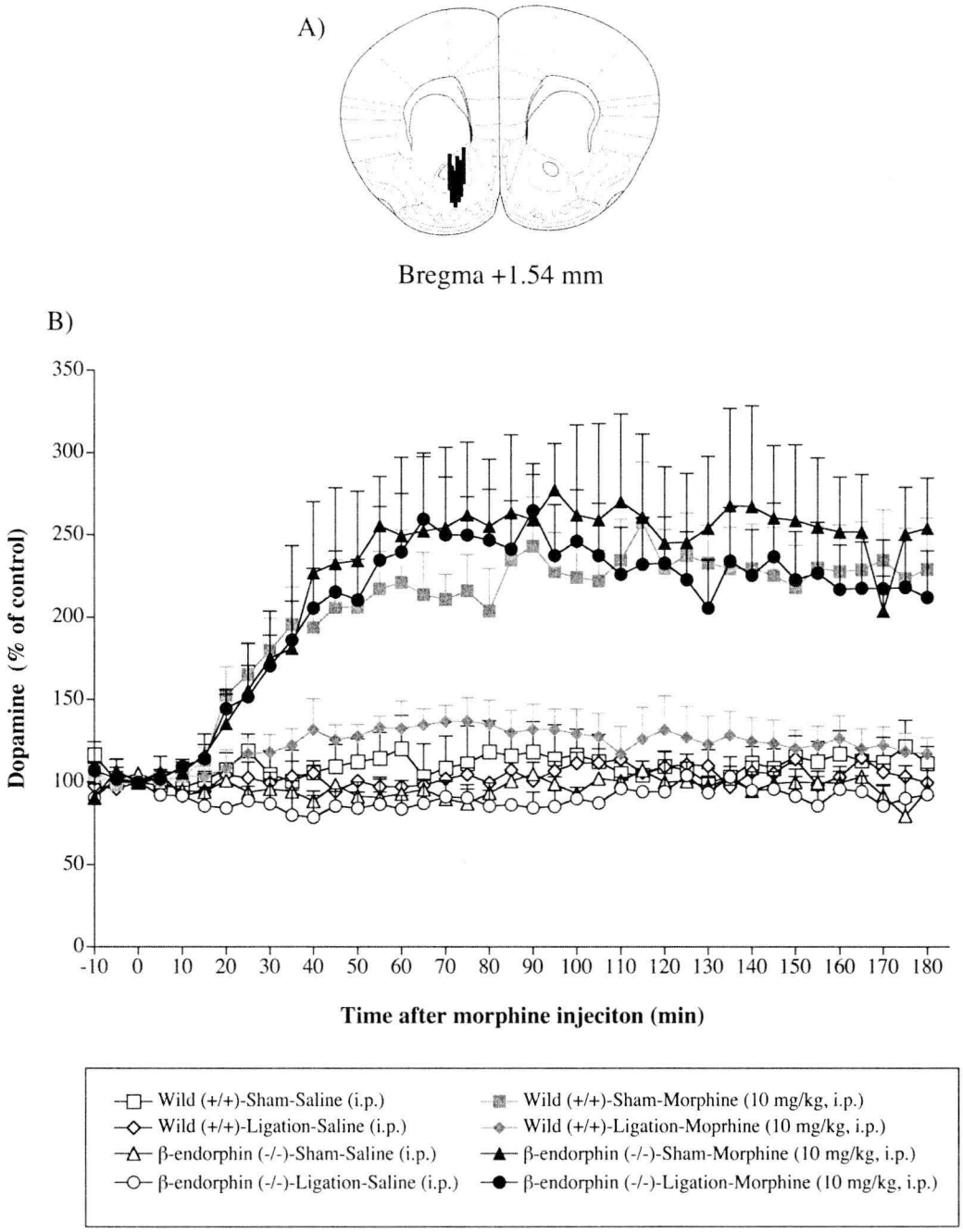


Fig. 4-7

Change in the increased dialysate dopamine level induced by morphine in β-endorphin knockout (–/–) mice. (A) Localization of microdialysis probe sites in the mouse N.Acc. Stippled lines represent regions in which microdialysis probes were inserted in the mouse brain. (B) Effects of treatment with morphine on the dialysate dopamine level in the N.Acc. in sham- or nerve-ligated wild-type and these β-endorphin knockout (–/–) mice. Morphine (10 mg/kg, s.c.) or saline was injected at time 0. The data are expressed as percentages of the corresponding baseline levels with S.E.M. of three to sevens mice. $F(1, 234) = 2.824$; $p<0.0001$, sham-operated wild-type mice treated with morphine vs. sham-operated β-endorphin knockout (–/–) mice treated with morphine.

Discussion

Previously, it has been reported that a state of neuropathic pain significantly suppressed the development of the rewarding effect produced by morphine in rodents, which was associated with a significant down-regulation of μ-opioid receptor function linked to an up-regulation of membrane-bound G-protein-coupled receptor kinase 2 in the VTA region, which possesses high densities of μ-opioid receptors [66,134]. This phenomenon strongly supports the clinical observation that psychological dependence on opioids is not a major problem for patients who suffer from a neuropathic pain-like state, including the pain associated with cancer. I proposed that a significant down-regulation of μ-opioid receptor function in this area may result from sustained activation of the endogenous μ-opioidergic system following nerve injury. I considered that nerve injury may consistently increase the release of endogenous opioid peptide that interacts with μ-opioid receptors in the VTA, resulting in a reduction of μ-opioid receptor function in this area. Several lines of evidence suggest that, in response to pain stimulus, the endogenous μ-opioid receptor agonist β-endorphin is released within some brain regions, including the mesolimbic area [127].

Thus, I thought that it might be better to directly measure β-endorphin released in the VTA of rodents with sciatic nerve ligation. However, some major problems had to be addressed if I were to directly prove this hypothesis. First, it was too hard to maintain an adequate experimental condition over a week with animals fixed to a cannula directly connected to sampling collectors. Second, the level of released

β-endorphin was expected to be so slight that I could have no confidence in detecting its content in the VTA area during a neuropathic pain-like state according to radioimmunoassay. Therefore, I decided to use both *in vivo* behavioral and biochemical approaches using a specific antibody to β-endorphin and β-endorphin gene knockout mice.

In the present study, I first confirmed that sciatic nerve ligation suppressed a place preference induced by microinjection of the selective μ-opioid receptor agonist DAMGO into the VTA in rats. Furthermore, I demonstrated that nerve injury reduced both the increase in the level of extracellular dopamine by s.c. morphine in the N.Acc. of wild-type mice and the increase in [^{35}S]GTPγS binding to membranes of VTA obtained from wild-type mice induced by DAMGO. Moreover, p-TH (Ser31) immunoreactivity in the rat VTA was dramatically diminished by sciatic nerve ligation, and some of p-TH-positive neurons were directly projected to the N.Acc. These findings provide further evidence that a neuropathic pain-like state causes a significant reduction in the activity of a μ-opioid receptor-mediated mesolimbic dopaminergic pathway in rodents.

Under these conditions, an intra-VTA injection of a specific antibody to β-endorphin reversed the suppression of the DAMGO-induced place preference by sciatic nerve ligation in rats. To further investigate the role of β-endorphin in the suppression of opioid reward under a neuropathic pain-like state, I next performed several experiments using β-endorphin gene knockout mice. It has been shown that the introduction of a stop codon in place of the tyrosine 179 codon in the proopiomelanocortin (POMC) gene

results in mutant mice that lack β-endorphin. These β-endorphin knockout mice exhibit normal morphine-induced analgesia. In addition, the expression of other peptide products (e.g. ACTH and MSH) from the POMC gene is not affected in these transgenic mice [110]. I hypothesized that the availability of these β-endorphin knockout mice could provide molecular evidence for the role of β-endorphin in the suppression of opioid reward under a neuropathic pain. Using these transgenic mice, I demonstrated here that the suppression of a morphine-induced place preference by sciatic nerve ligation was abolished in β-endorphin gene knockout mice, indicating that endogenous β-endorphin is required for recovery from the suppression of opioid reward under a neuropathic pain-like state in rodents. The present result was mostly consistent with the recent finding by Petraschka et al. (2007) [135].

A key finding in the present study is that the increased [^{35}S]GTPγS binding by DAMGO to membranes in the VTA area obtained from wild-type mice was dramatically decreased by sciatic nerve ligation and this effect was abolished in mice that lacked the β-endorphin gene following sciatic nerve ligation. These results provide molecular evidence that endogenous β-endorphin corresponds to the down-regulation of μ-opioid receptor function to activate G-proteins in the VTA following nerve injury. Therefore, I propose here that β-endorphin released by chronic nociceptive stimuli may continuously activate μ-opioid receptors in the VTA and in turn lead to the down-regulation of μ-opioid receptor function. This phenomenon may be directly involved in reduced opioid reward under a neuropathic pain-like state in rodents.

Another key finding of the present study is that the increased extracellular dopamine in the N.Acc. produced by s.c. treatment with morphine was diminished by sciatic nerve ligation in wild-type mice, and this effect was also eliminated in β-endorphin knockout mice with sciatic nerve ligation. This result strongly supports the idea that endogenous β-endorphin is responsible for the suppression of opioid reward linked to a mesolimbic dopaminergic system under a neuropathic pain-like state.

In conclusion, I demonstrated here that suppression of a μ-opioid-induced place preference by sciatic nerve ligation was abolished by microinjection of the β-endorphin antibody into the VTA and deletion of the β-endorphin gene. Furthermore, the deletion of β-endorphin also eliminated the reductions in both μ-opioid receptor function in the VTA by nerve ligation and in the activity of mesolimbic dopaminergic transmission projecting from the VTA to the N.Acc. regulated by μ-opioid receptors in the VTA. These findings provide further evidence that β-endorphin released in the VTA is a key player in regulating the dysfunction of μ-opioid receptor function to modulate opioid reward under a neuropathic pain-like state.

General Conclusion

The above findings led to the following conclusions:

In Chapter 1:

In the present study, I found that a PAR-1 and PDGF-A-mediated signaling pathway within spinal cord neurons may be directly implicated in neuropathic pain after nerve injury in mice. Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were significantly suppressed by repeated i.t. injection of hirudin, which is a specific and potent thrombin inhibitor. Furthermore, a single i.t. injection of thrombin produced long-lasting hyperalgesia and allodynia, and these effects were also inhibited by hirudin in normal mice. In immunohistochemical studies, increased PAR-1-like IR in the spinal cord after nerve injury was co-localized with PKCγ. In addition, thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were also suppressed by repeated i.t. injection of either the PDGF α receptor (PDGFRα)/Fc chimera protein or the PDGFR-dependent PTK inhibitor AG17. Moreover, thermal hyperalgesia and tactile allodynia induced by thrombin in normal mice were virtually eliminated by i.t. pretreatment with PDGFRα/Fc.

The present data provide novel evidence that a PAR-1 and PDGF-A-mediated signaling pathway within the spinal cord is directly involved in the development of the neuropathic pain-like state induced by sciatic nerve ligation in mice. Furthermore, this signal pathway may lead to PKCγ activation. Such findings raise the fascinating

possibility that the activation of up-regulated PAR-1 located on spinal dorsal horn neurons following nerve injury may release PDGF in the spinal cord and in turn activate PDGFRα, leading to a neuropathic pain-like state.

In Chapter 2:

The activation of spinal PKC by i.t. treatment with PDBu caused a remarkable increase in the activity of several brain regions in wild-type mice compared with vehicle treatment using fMRI assay. In the somatosensory cortex, lateral thalamic nuclei and medial thalamic nuclei, which are sensory-discriminative components of pain, i.t. injection of PDBu produced a dramatic and time-dependent increase in signal intensity. In contrast, i.t. injection of PDBu produced a delayed but significant increase in signal intensity in the cingulate cortex, nucleus accumbens and ventral tegmental area, which are affective-motivational components of pain. Furthermore, these effects of PDBu were abolished in mice that lacked the PKCγ gene. These results suggest that the activation of spinal PKCγ associated with the activation of ascending pain transmission may be an important factor in chronic pain-like hyperalgesia with changes in emotionality.

In Chapter 3:

In the present study, I found that the activation of spinal PKC by i.t. treatment with PDBu, a specific PKC activator, caused thermal hyperalgesia, pain-like behaviors and suppression of the morphine-induced rewarding effect. This suppression of morphine

reward was eliminated in mice that lacked β-endorphin. In contrast, thermal hyperalgesia and pain-like behaviors were not affected in β-endorphin knockout mice. These results suggest that the activation of PKC in the spinal cord may play an essential role in the suppression of the morphine-induced rewarding effect in mice with neuropathic pain through the constant release of β-endorphin.

In Chapter 4:

I confirmed that sciatic nerve ligation suppressed a place preference induced by DAMGO and reduced both the increase in the level of extracellular dopamine by s.c. morphine in the nucleus accumbens and [^{35}S]GTPγS binding to membranes of the VTA induced by DAMGO. Interestingly, these effects were eliminated in mice that lacked the β-endorphin gene. Furthermore, intra-VTA injection of a specific antibody to the endogenous μ-opioid peptide β-endorphin reversed the suppression of the DAMGO-induced rewarding effect by sciatic nerve ligation in rats. These results provide molecular evidence that nerve injury results in the continuous release of endogenous β-endorphin and in turn the dysfunction of μ-opioid receptors in the VTA. This phenomenon could explain the mechanism that underlies the suppression of opioid reward under a neuropathic pain-like state (See Fig. A).

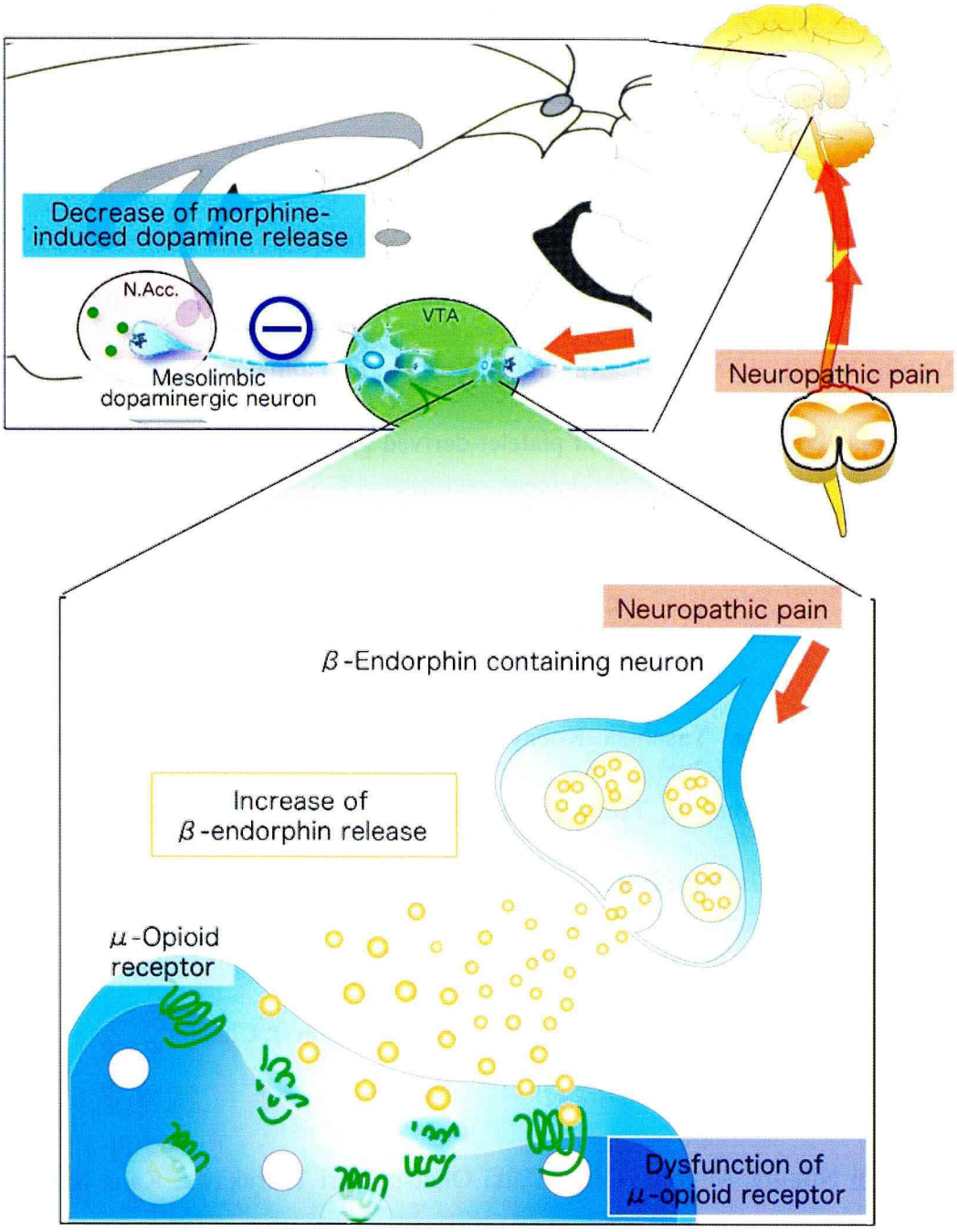


Fig.A
Schematic illustration of mechanism on the suppression of morphine-induced rewarding effect under a neuropathic pain-like state.

List of Publications

This dissertation is based on the following original publications:

1) Minoru Narita, Aiko Usui, Michiko Narita, Keiichi Niikura, Hiroyuki Nozaki, Junaidi Khotib, Yasuyuki Nagumo, Yoshinori Yajima and Tsutomu Suzuki: Protease-activated receptor-1 and platelet-derived growth factor in spinal cord neurons are implicated in neuropathic pain after nerve injury. J. Neurosci., 25, 10000 –10009 (2005): Chapter 1

2) Keiichi Niikura, Yasuhisa Kobayashi, Daiki Okutsu, Masaharu Furuya, Kumi Kawano, Yoshie Maitani, Tsutomu Suzuki and Minoru Narita: Implication of spinal protein kinase Cγ isoform in activation of the mouse brain by intrathecal injection of the protein kinase C activator phorbol 12,13-dibutyrate using functional magnetic resonance imaging analysis. Neuroscience Letters (*in press*): Chapter 2

3) Keiichi Niikura, Minoru Narita, Daiki Okutsu, Yuri Tsurukawa, Kana Nanjo, Kana Kurahashi, Yasuhisa Kobayashi and Tsutomu Suzuki: Implication of endogenous β-endorphin in the inhibition of the morphine-induced rewarding effect by the direct activation of spinal protein kinase C in mice. Neuroscience Letters (*in press*): Chapter 3

4) Keiichi Niikura, Minoru Narita, Michiko Narita, Atsushi Nakamura, Daiki Okutsu, Ayumi Ozeki, Kana Kurahashi, Yasuhisa Kobayashi, Masami Suzuki and Tsutomu Suzuki: Direct evidence for the involvement of endogenous β-endorphin in the suppression of the morphine-induced rewarding effect under a neuropathic pain-like state. Neuroscience Letters (*in press*): Chapter 4

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